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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [7] : A61K 38/00	A2	(11) International Publication Number: **WO 00/25804** (43) International Publication Date: 11 May 2000 (11.05.00)

(21) International Application Number: PCT/US99/25692

(22) International Filing Date: 2 November 1999 (02.11.99)

(30) Priority Data:
60/106,718   2 November 1998 (02.11.98)   US
60/145,883   27 July 1999 (27.07.99)   US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: A METHOD FOR INHIBITION OF PHOSPHOLAMBAN ACTIVITY FOR THE TREATMENT OF CARDIAC DISEASE AND HEART FAILURE

(57) Abstract

The present invention provides a method for the treatment of heart failure through the use of small peptide complexes and recombinant proteins which function to enhance contractility in failing hearts and reduce blood pressure in individuals with hypertension by inhibiting the interaction between phospholamban and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) within cardiomyocytes. In addition, a means is provided for the transport of such therapeutic agents into the cytoplasm and nucleus of cardiomyocytes.

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A METHOD FOR INHIBITION OF PHOSPHOLAMBAN ACTIVITY FOR THE TREATMENT OF CARDIAC DISEASE AND HEART FAILURE

This application claims the benefit of priority of United States

5   Provisional Application Serial No. 60/106,718, filed November 2, 1998

and United States Provisional Application Serial No. 60/145,883, filed

July 27, 1999, both of which are incorporated herein by reference in their

entirety.

10   **BACKGROUND OF THE INVENTION**

FIELD OF THE INVENTION

This invention relates generally to a method for the treatment of

heart failure, and more specifically to the inhibition of phospholamban

(PLB) activity for the treatment of myocardial dysfunction.

15

BACKGROUND INFORMATION

Heart failure is the leading cause of combined morbidity and

mortality in the United States and other developed countries. Congestive

heart failure is characterized by a reduced contraction and delayed

20   relaxation of the heart however, fundamental molecular mechanisms

which drive the patho-physiological pathways for congestive heart are

largely unknown. Current therapy for the heart disease is primarily

palliative and is not targeted to the underlying biological pathways which

are thought to lead to the initiation and progression of cardiac muscle

25   dysfunction.

Heart muscle failure is a complex, integrative, multi-factorial disease

in which the genetic pathways that confer susceptibility are interwoven

with the environmental stimulus of biomechanical stress that accompanies

heart injury, pressure and volume overload, and genetic defects in

30   components of the cytoskeleton. In response to this biomechanical

stress, a series of parallel and converging signaling pathways are activated
that lead to the adaptive response of compensatory hypertrophy.
Subsequently, there can be a transition to chamber dilation and pump
failure that is associated with a loss of viable myocytes, a decrease in
5   contractile elements, myofilament disarray and interstitial fibrosis.

Recently, the activation of signal transduction pathways which
trigger the onset of programmed cell death have been implicated in
promoting the pathological transition to heart failure, as well as a gp130
dependent myocyte survival pathway that can block the actions of the
10   pro-apoptotic pathways and prevent the early onset of heart failure and
cardiomyopathy. In addition to these extrinsic stress-related pathways for
myocyte adaptation, there also must be intrinsic signaling pathways that
lead to the impairment of cardiac excitation-contraction (EC) coupling and
associated severe defects in cardiac contractile performance that are the
15   clinical hallmarks of the progression of the heart failure phenotype.

The sarcoplasmic reticulum (SR) plays an integral role in the
coordination of the movement of cytostolic Ca^{2+} throughout the cardiac
tissue. In separate studies by Mercadier, *et al.* (*J. Clin. Invest.*,1990;
85:305-309), Arai, *et al.* (*Circ. Res.*, 1993; 72:463-469), de la Bastie, *et*
20   *al.* (*Circ. Res.*, 1990; 66:554-564), and Feldman, *et al.* (*Circ. Res.*, 1993;
73:184-192), research on human failing hearts and animal models of heart
failure have suggested that the reduced uptake the cytostolic Ca^{2+} by the
SR is responsible for prolonged diastolic relaxation. Ca^{2+} stored in the SR
is released into the cytosol to activate the contraction of cardiac muscle
25   and subsequently re-accumulated to achieve relaxation. Activity of the
cardiac SR Ca^{2+} ATPase (SERCA2a) is the rate determining factor of Ca^{2+}
re-uptake into the SR, and SERCA2a activity is itself regulated by
phospholamban, a 52-amino acid muscle-specific SR phosphoprotein.

Phospholamban (PLB) was first identified as a major
30   phosphorylation target in the SR membrane in research by Tada, *et al.* (*J.*

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Bio.l Chem., 1974; 249:6174-6180) and appeared to be a potent inhibitor
of SERCA2a activity in its unphosphorylated form. The inhibitory effect
of PLB on SERCA2a is reduced by an increase in intracellular calcium or by
the phosphorylation of PLB in response to β-adrenergic stimulation. PLB

5   exists primarily in a pentameric form, that when subjected to high
temperature, dissociates into five equivalent monomers.

The amino acids of monomeric PLB are grouped into three physical
and functional domains. Domains Ia and II are rich in α-helices and are
connected by the less structured domain Ib. Domain Ia is composed of

10   amino acids 1-20, the majority of which are in an α-helical confirmation,
having a net positive charge. Domain Ib consists of amino acid residues
21-30 and constitutes the cytoplasmic sector of the monomer. Domain II
amino acids 31-52, represents the transmembrane sector and is made up
solely of uncharged residues that are responsible for stabilizing the

15   pentamer formation.

PLB is a mediator in the regulation of myocardial function by
catecholamines through the cAMP cascade. Ser (16) and Thr (17), in
domain Ia are the confirmed binding sites for cAMP-dependent protein
kinase (PKA) and Ca/calmodulin-dependent protein kinase, respectively,

20   which function to catalyze phosphoester phosphorylation of PLB which in
turn relieves its inhibition on SERCA2a activity. Because Ser (16) and Thr
(17) can be phosphorylated by the kinases, the net charge of the amino
acids can be shifted from positive to neutral and even to negative.
Together with the charged residues of SERCA2a, the shifting of charges

25   in domain Ia of PLB can result in profound alterations in the protein-protein
interaction of the PLB-SERCA2a system. Domain II also contains some
key amino acids for functional expression of PLB, in that amino acids of
one face of the domain II helix are associated with the transmembrane
domain of SERCA2a.

30   There may be two ways in which PLB regulates Ca^{2+}-ATPase

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activity: 1) a quick-acting, short-term mechanism involving PLB
phosphorylation and depression of calcium pumping activity, and 2) a
slower acting but longer term process involving a change in the molecular
ratio of PLB to the Ca^{2+}-ATPase brought about by control of gene

5   expression. Under physiological conditions, phosphorylation at Ser (16)
by PKA is the predominant event that leads to proportional increases in
the rate of Ca^{2+} uptake to the SR and accelerates ventricular relaxation.
An increase in the relative ratio of PLB to SERCA2a is an important
determinant of SR dysfunction in both experimental and human heart

10 failure. Moreover, attenuated PLB phosphorylation by PKA may be
responsible for impaired diastolic function and prolonged Ca^{2+} transients
in failing hearts by which the β-adrenergic receptor-cAMP system is
severely down-regulated by enhanced sympathetic tone.

A detailed mutagenesis study by Toyofuku, et al. (J. Biol. Chem.,

15 1994; 269:3088-3094) revealed that several amino acids in the
cytoplasmic domain of PLB are important for its inhibitory function. The
study showed that when the certain amino acids were mutated into amino
acids of different charge, the PLB mutants lost their inhibitory effect on
the co-transfected SERCA2 in HEK293 cells. However, it is still unclear

20 whether PLB bearing these mutants can exert dominant negative effects
on endogenous wild-type PLB and consequently stimulate endogenous
SERCA2a in cardiac myocytes. Additionally, it is unclear how the
mechanisms of transfer of these PLB point mutations into cardiomyocytes
breach the cytoplasmic membrane barrier in order to effect endogenous

25 SERCA2a activity.

Genetically based mouse models of dilated cardiomyopathy by
Arber, et al. (Cell, 88:393-403; 1997) provide evidence that chamber
dilation and the progression to heart failure is dependent on a specific
Ca^{2+} cycling defect in the cardiac sarcoplasmic reticulum. In the mouse

30 models, ablation of phospholamban (PLB) rescued the spectrum of

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structural, functional, and molecular phenotypes that resemble heart failure. Furthermore, release of the phospholamban-SERCA2a interaction through the forced *in vivo* overexpression of a PLB point mutation dominantly activated the contractility of ventricular muscle cells. Thus,

5   there is the possibility that interfering with the PLB-SERCA2a interaction may provide a novel therapeutic approach for preventing heart failure.

There is the understanding that interfering with the PLB-SERCA2a interaction may be a potential therapeutic target for the treatment of heart failure, however, the internalization of exogenous molecules to enhance

10   cardiac contractility by live myocytes remains an unsolved issue. A means must be available to deliver any therapeutic agent directly to the cytoplasm and nucleus of cardiac myocytes. Penetratins, a class of peptides with translocating properties, have the ability to carry hydrophillic compounds across the plasma membrane. Research by Schwarze, *et al.*

15   (*Science* 285:1569-1572; 1999) has demonstrated an approach to protein transduction using a penetratin-based fusion protein which contains an NH_2-terminal 11- amino acid protein transduction domain from the denatured HIV TAT protein (Genebank Accession No. AF033819) . Using this non-cell-type specific transfer system allows direct targeting of

20   oligopeptides and oligonucleotides to the cytoplasm and nucleus. One of the most well characterized translocation peptides is one that corresponds to residues 43 to 58 of antennapedia, a Drosophila transcription factor. It is believed that the translocation peptide interacts with charged phospholipids on the outer side of the cell membrane. Destabilization of

25   the bilayer results in the formation of inverted micelles containing the peptide that travels across the cell membrane and eventually open on the cytoplasmic side. While the use of transport peptides to move cargo molecules into cells is not novel, it has not been demonstrated that transport peptides work well in cardiomyocytes.

30   Thus the need remains for methods for the inhibition of PLB through

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the use of mutants or small molecule inhibitors of PLB in order to manipulate the PLB/SERCA2a interaction in cardiac myocytes, as well as a transport means for these mutants or small molecule inhibitors of PLB to cross sarcoplasmic reticulum membrane barriers into the cytoplasm of

5   cardiac myocytes for the treatment of cardiac disease and heart failure. The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

10   It is an advantage of the present invention to provide methods for treatment of heart failure by inhibiting the effect of phospholamban on Ca^{2+} uptake in cardiac tissue.

It is another advantage of the present invention to provide both small peptide complexes and recombinant proteins which function to

15   enhance contractility in failing hearts and reduce blood pressure in individuals with hypertension by inhibiting the interaction between phospholamban and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) within cardiomyocytes.

It is yet another advantage of the present invention to provide for a

20   family of compounds that consist of a transport peptide covalently attached to wild-type, mutant, or truncated PLB.

In a first exemplary embodiment of the present invention, recombinant adenoviruses are engineered which force the expression of wild-type or mutant forms of PLB which have the ability to selectively

25   interrupt the normal inhibitory interaction between PLB and SERCA2a and in turn dominantly activate cardiac contractility.

In a second exemplary embodiment of the present invention, contractilin, a recombinant adenoviral mutant of PLB (K3E/R14E), binds to and imitates phosphorylation of phospholamban. This leads to an

30   activation of the calcium pump of the sarcoplasmic reticulum thus

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increasing cardiac contractility.

In a third exemplary embodiment of the present invention, a compound consisting of a fusion of 1) a 16-residue transport peptide and 2) a truncated phospholamban protein or similar peptide are transported

5   across the cell membranes in a receptor independent manner. Once inside the cytoplasm of the cardiomyocyte, the truncated phospholamban or similar peptide act as a competitive inhibitor of endogenous phospholamban interactions with SERCA2a.

10   **BRIEF DESCRIPTION OF DRAWINGS**

Figure 1 is an illustration diagraming a working model for the role of the PLB-SERCA2a interaction in the progression of heart failure.

Figure 2 shows the hemodynamic analysis of rescue of in vivo cardiac dysfunction in DKO mice (a-d) and hemodynamic assessment of β-

15   adrenergic response to progressive infusion of dobutamine (e-h), where Figure 2a shows the plot for maximal first derivative of LV pressure, LV dP/dtmax. Figure 2b shows the plot for minimal first derivative of LV pressure, LV dP/dtmin. Figure 2c shows the plot for Lv end diastolic pressure. Figure 2d shows the plot for Tau. Figure 2e shows a graph of

20   maximal first derivative of LV pressure, LV dP/dtmax. Figure 2f shows a graph of minimal first derivative of LV pressure, LV dP/dtmin. Figure 2g shows a graph of Lv end diastolic pressure. Figure 2h shows a graph of heart rate.

Figure 3 shows plotted data from the analysis of rescue of

25   physiological calcium signaling defects in DKO myocytes, where Figure 3a is a series of graphs of representative intracellular Ca^{2+} transient in WT, MLPKO and DKO myocytes. Figure 3b is a bar graph of the amplitude of Ca^{2+} transients. Figure 3c is a bar graph of intracellular diastolic Ca^{2+} concentration. Figure 3d is a bar graph of SR Ca^{2+} content.

30   Figure 3e is the immunoblot of MLP deficiency.

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Figure 4a shows a dot blot analysis of rescue of embryonic gene markers of the heart failure phenotype in DKO mice. Figure 4b is a bar graph comparing the relative induction of mRNA for wild-type, MLPKO, and DKO myocytes.

5   Figure 5 illustrates the inhibition of the interaction between PLB and SERCA2a, where Figure 5a is a series of graphs which plot the length change in myocytes. Figure 5b is a summary of the data of cell length changes.

Figures 6a is a Western blot analysis of myocytes containing the

10   adenovirus transgenes expressing sense PLB (sPLB), antisense PLB (asPLB), E2A, R14E, S16N, and K3E/R14E against monoclonal PLB. Figure 6b is a Western blot showing the results of cell infectivity by PLB, sPLB and K3E/R14E. Figure 6c illustrates the Western blot analysis of PLB infectivity of Sol8 cells.

15   Figure 7 shows a plot of SR Ca 2 + uptake in homogenates of neonatal rat cardiomyocytes infected with adenovirus expressing the indicated genes.

Figure 8 shows a plot of the data derived from indo 1 fluorescence-facilitated Ca 2 + transients of myocytes.

20

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

To directly assess the role Ca^{2+} cycling defects play in the transition to heart failure, cardiomyopathy in muscle-specific LIM protein (MLP)-deficient mice can be reversed by removing the gene that codes for

25   PLB. Mice which harbor an ablation of MLP show many of the phenotypic features of human dilated cardiomyopathy at the molecular, cellular, and physiological levels. A uniform feature of end-stage dilated cardio-myopathy is a marked increase is cardiac wall stress that is accompanied by thinning of the chamber wall and an accompanying decrease in cardiac

30   contractility and relaxation. Because calcium cycling is critical for both

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cardiac relaxation and contractility, defects in the pathway that control calcium uptake and release from the sarcoplasmic reticulum are prime candidates for driving the progression of heart failure.

5   The creation of mice which harbor a deficiency in PLB, in addition to MLP, exhibit rescue from all the phenotypic characteristics of human heart failure normally found in MLP single knock-out (MLPKO) mice. To determine whether the functional benefits associated with PLB ablation in MLPKO mice specifically reflect the loss of the direct interaction between PLB and SERCA2a, the applicant of the present invention has engineered

10   point mutations in the PLB gene which interrupt the functional inhibitory interaction between PLB and SERCA2a. Through the creation of recombinant adenoviruses encoding point mutations in PLB, it is demonstrated that progressive defects in excitation-contraction coupling in heart failure are related to the enhanced inhibition of SERCA2a by PLB

15   and that the introduction of phospholamban deficiency into the setting of a transgenic model of cardiac hypertrophy results in rescued cardiac function. These results are independently supported by the fact that MLP-deficient mice harboring a transgene which directs cardiac specific overexpression of SERCA2a also exhibit rescue of the cardiomyopathy

20   phenotype. Taken together, these results provide clear evidence that sarcoplasmic reticulum calcium cycling is critical to the progression of heart failure and points to the critical regulatory role of PLB inhibition of SERCA2a activity in the progression of heart failure. This, in turn, pinpoints the possibility of PLB as a key therapeutic target.

25   Further study of the MLP-PLB knock-out (DKO) mice indicated that the induction of PLB deficiency in the setting of cardiomyopathic mutation can result in maximal stimulation of cardiac contractile performance. The contractility of the DKO hearts at baseline levels was comparable to the contractility of wild-type hearts following maximal β-adrenergic

30   stimulation. This result suggests that following the removal of the tonic

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inhibition of SR calcium pump function by PLB, there is essentially a "supra-rescue" in terms of cardiac contractile function of the cardiomyopathic heart. Since PLB is a direct substrate for phosphorylation by both cyclic AMP-dependent protein kinase A and calcium/calmodulin

5   dependent kinase, the regulation of cardiac contractility by cAMP-dependent stimuli may occur via the phosphorylation of PLB, which in turn prevents the inhibitory interaction with SERCA2a.

According to the theory behind the phosphorylation of PLB, the "supra-rescue" of the cardiomyopathic MLP-deficient mice to super-normal

10   levels in the setting of PLB-deficiency might simply reflect the removal of the rate limiting step in the tonic inhibition of cardiac contractility. Consistent with this rationale, studies by Rockman, et al. (Proc. Natl. Acad. Sci. USA 95:7000-7005; 1998) have documented that relief of β-adrenergic desensitization in the MLP-deficient mice can also lead to

15   significant rescue of the dilated cardiomyopathic phenotype. Since PLB is an SR protein that interacts with at least three regulatory components (cAMP-dependent protein kinase, calcium/calmodulin-dependent kinase, and protein phosphatase), it should be determined whether the dominant effect of PLB ablation on improving cardiac contractile performance

20   reflects the chronic interaction of PLB with the SERCA2a or whether this rescue effect is related to the interaction of PLB with other known or novel cardiac proteins.

Utilizing recombinant adenoviruses which force the expression of wild-type and mutant forms of PLB, the present invention provides for

25   point mutations in PLB that can selectively interrupt the normal inhibitory interaction between PLB and SERCA2a and can dominantly activate cardiac contractility in cardiac ventricular muscle cells in the absence of catecholamine. Figure 1 outlines the mechanism responsible for the rescue effect observed in the DKO mice. Both PLB and MLP are muscle-

30   specific proteins, and as such, there must be a muscle cell autonomous

pathway that is required for the progression and the rescue of the
phenotype, as opposed to extrinsic stress signals that promote or
suppress myocyte survival pathways. Since PLB and MLP do not directly
interact at the protein level, eliminating direct interaction between PLB and
5   MLP as the basis for rescue, there must be a physiological as opposed to
biochemical regulatory pathway that links the PLB regulatory pathways
with the onset of dilated cardiomyopathy. As shown in Figure 1, in the
normal heart, SR-calcium stores are maintained through the activity of the
SERCA2a which leads to an uptake of calcium into the SR and
10   consequent maintenance of normal cardiac relaxation and reduction in wall
stress. Subsequently, SR release of calcium, via the calcium release
channel, results in normal quantal calcium release and the consequent
activation of the cardiac myofilaments leading to myocardial contraction.
Accordingly, enhanced Ca^{2+} content in the SR leads to an enhanced Ca^{2+}
15   release with a corresponding increase in cardiac contractility.

The activity of SERCA2a is regulated by the inhibitory effects of the
direct interaction of PLB with SERCA2a, which can be relieved by the
cAMP-dependent phosphorylation of PLB following the delivery of β-
adrenergic stimuli. In the setting of heart failure, there is a relative
20   decrease in SERCA2a function due to an inhibitory effect of PLB that
arises due to blunted β-adrenergic responsiveness. As a result, there is
less phosphorylation of PLB and a constitutive inhibition of SERCA2a, via
chronic interaction with PLB, leading to a relative decrease is SR calcium
content versus normal levels. This decrease in calcium stores is
25   translated into a decrease in the quantal release of calcium through the
calcium release channel and a consequent decrease in the single cell
calcium transients and in vivo cardiac contractility. In the DKO mice, the
inhibitory effect of PLB is removed, as shown in Figure 1, thereby
relieving the system from the downstream inhibitory effects of PLB on the
30   SR calcium pump, resulting in maintenance of SR calcium uptake and

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reduction of wall stress towards normal levels. At the same time, this increase in SR calcium content results in maintenance of normal calcium quantal release, thereby leading to maintenance of normal contractility and relaxation.

5

Muscle-specific LIM protein knock-out and Double knock-out mice

Tests of the present invention were conducted using a double knock-out (DKO) mouse model which harbors homozygous ablation of two independent muscle specific genes. For this strategy, PLB knock-out
10   (PLBKO) mice are mated into the background of MLP knock-out (MLPKO) mice which harbor molecular, structural and physiological features of the complex *in vivo* heart failure phenotype of dilated cardiomyopathy. The F3 generation of these mice are used for the actual experimentation to eliminate any potential background effects from either the PLBKO or
15   MLPKO line on the observed cardiac phenotype of the DKO line.

MLPKO mice display a marked increase in heart/body weight ratio (6.34 $\pm$ 0.22 mg/g, n = 8) versus age and gender matched wild-type mice (4.60 $\pm$ 0.21 mg/g, n = 7; p < 0.001). The heart/body weight ratio in DKO mice (5.13 $\pm$ 0.19 mg/g, n = 9) is significantly smaller than that of
20   MLPKO mice (p < 0.01) and is not statistically different from wild-type. To evaluate whether the decreased heart weight in DKO mice is associated with amelioration of the disrupted cytoskeletal phenotype observed in MLPKO mice, electron microscopic analysis is made of hearts from MLPKO and DKO littermates. Ablation of PLB in the background of
25   MLP $^{-/-}$ rescues the wide spectrum of ultrastructural defects originally observed in the MLP deficient hearts, including myofibrillar disarray and massive fibrosis. These data suggest that ablation of PLB prevents not only the increase in total heart mass, but also prevents the disorganization of cardiomyocyte cytoarchitecture and fibrosis in MLPKO cardiomyopathic
30   mice.

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To evaluate whether the marked decreases observed in *in vivo*
global cardiac function is rescued in DKO mice, echocardiography is
performed with age-matched littermates. As noted in Table 1, prevention
of ventricular dilation is confirmed in DKO mice. MLPKO mice have
5   enlarged cardiac chambers, as revealed by increased left ventricular end-
diastolic dimensions (LVEDD) and end-systolic dimensions (LVESD),
whereas DKO mice have LVEDD in the normal range. The percent
fractional shortening (%FS) and mean velocity of circumferential fiber
shortening (mean Vcf), indicators of systolic cardiac function, are
10   improved in age-matched DKO littermates, also noted in Table 1. When
compared to non-littermate wild-type mice as controls, most of the
echocardiographic data in DKO mice are similar to those in wild-type mice,
although %FS is slightly decreased in DKO mice. Furthermore, cardiac
function of DKO mice remain in the normal range beyond 6 months of age
15   (n = 2). Despite the reduction of chamber dilation, there appears to be
some hypertrophy in DKO hearts. The ratio of LVEDD to LV posterior wall
thickness is markedly decreased in DKO mice, indicating that the wall
stress of DKO mice is reduced in comparison to MLPKO or wild-type mice.
These results indicate that global cardiac function of DKO mice is
20   preserved in the range comparable to the parameters of control hearts. It
should be noted that mice which are heterozygous for PLB deficiency
display an intermediate level of functional rescue versus the MLPKO and
DKO mice, suggesting that partial ablation of PLB can lead to significant
functional improvement of the heart failure phenotype in MLPKO mice.

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Table 1

Echocardiographic assessment

	MLPKO n = 12	MLPKO/PLBhet n = 12	DKO n = 10	wild-type n = 10
LVEDD (mm)	4.87 ± 0.14	4.10 ± 0.15***	3.75 ± 0.12***	3.89 ± 0.11***
LVESD (mm)	3.94 ± 0.18	3.06 ± 0.22*** †	2.60 ± 0.10***	2.44 ± 0.07***
FS(%)	19.4 ± 1.7	26.2 ± 3.2* ‡	30.6 ± 1.8*** †	37.2 ± 1.4***
SEPth (mm)	0.68 ± 0.05	0.86 ± 0.05* ‡	0.83 ± 0.05* ‡	0.64 ± 0.01
Pwth (mm)	0.67 ± 0.05	0.86 ± 0.05* ‡	0.81 ± 0.04* †	0.64 ± 0.06
LVEDD/ Pwth(mm/mm)	7.47 ± 0.30	5.00 ± 0.47***	4.74 ± 0.30***	6.14 ± 0.18*
mean Vcf (circ/s)	3.38 ± 0.33	4.81 ± 0.73#	6.10 ± 0.41*** †	4.41 ± 0.61
mean Vcfc (circ/s)	1.41 ± 0.17	1.96 ± 0.27*	2.37 ± 0.13***	2.23 ± 0.10**
Heart Rate (beats/min)	351 ± 15	350 ± 15§	396 ± 13§	239 ± 14***
Age (days)	65.3 ± 2.5	65.5 ± 4.5‡	67.4 ± 1.9‡	80.5 ± 1.2**
Body Weight (g)	26.7 ± 1.3	26.8 ± 0.9§	25.0 ± 1.3§	36.9 ± 2.2***

MLPKO vs. MLPKO/PLBhet or DKO or wild-type; *: p<0.05, **: p<0.01, ***: p<0.001

wild-type vs. MLPKO/PLBhet or DKO; †: p<0.05, ‡: p<0.01, §: p<0.001

MLPKO/ PLBhet vs. DKO; #: p<0.05

MLPKO mice have demonstrated a marked blunting of β-adrenergic responsiveness and decreased adenyl cyclase activity. Ablation of PLB by homologous recombination in mice can augment cardiac contractile performance to a level comparable to that with maximal β-adrenergic stimulation of the normal heart. To confirm that the ablation of PLB can reverse the hemodynamic defects and marked β-adrenergic receptor desensitization associated with dilated cardiomyopathy, anesthetized mice are cardiac catheterized and assessed.

Several independent hemodynamic parameters document the rescue of the severe cardiac dysfunction with circulatory congestion to normal levels in the DKO mice. LV contractility (assessed by LV dP/dtmax) and

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relaxation (assessed by LV dP/dtmin) at baseline appears to be higher than that of wild-type mice and is comparable to PLBKO mice, as is shown in Figures 2a and 2b. Ablation of PLB reverses the markedly elevated LV end diastolic pressure observed in the MLPKO cardiomyopathic mice, as

5   seen in Figure 2c.

Analysis of Figure 2d illustrates that Tau, an indicator of LV relaxation and diastolic function, are also normalized in the DKO mice, which is consistent with an improvement in wall stress. These data suggest that ablation of PLB can rescue both the systolic and diastolic

10   dysfunction in the cardiomyopathic MLPKO mice. The blunted responses of LV dP/dtmax and LV dP/dtmin to β-adrenergic stimulation is observed in MLPKO mice, as shown in Figures 2e and 2f, indicating the presence of severe β-adrenergic desensitization in MLPKO mice. There is no stimulation of cardiac contractility (LV dP/dtmax) and relaxation (LV

15   dP/dtmin) by dobutamine in DKO mice, as is again shown in Figures 2e and 2f. These parameters are already stimulated to their maximal levels under basal conditions in the DKO hearts.

After maximum stimulation of wild-type hearts by dobutamine, LV dP/dtmax and LV dP/dtmin are indistinguishable from these parameters in

20   the DKO mice in the absence of any catecholamine stimulation. This evidence confirms that the interaction of PLB and SERCA2a suppresses cardiac contractility in both normal and myopathic hearts, and that inhibiting this interaction may exert a dominant effect on maximizing cardiac performance in the absence of any catecholamine stimulation.

25   Figure 2h shows that chronotropic responsiveness to dobutamine is preserved in both DKO mice and MLPKO mice, thereby documenting the specificity of the β-adrenergic response to ventricular myocytes versus pacemaker cell types.

To determine the mechanisms responsible for the rescue of *in vivo*

30   cardiac function in DKO mice, several independent parameters of Ca^{2+}

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signaling are assessed. It is apparent that altered Ca^{2+} homeostasis in
DKO mice leads to the hemodynamic changes, intracellular Ca^{2+} transients
and the expression of proteins related to Ca^{2+} cycling in the SR. MLPKO
myocytes exhibit an attenuated amplitude of Ca^{2+} transients with normal
5   levels of diastolic Ca^{2+} concentration, as is show in Figures 3a-c. The
rate of decay is slightly accelerated in MLPKO mice which suggests that a
compensatory mechanism is operative during the end of Ca^{2+} uptake in
MLPKO mice. Ablation of PLB is associated with a shortened duration of
the Ca^{2+} transient, faster decay and preserved amplitude. Figure 3d
10   shows that SR Ca^{2+} content is significantly decreased in MLPKO mice and
increased in DKO mice as compared to wild-type mice. Quantitative
immunoblotting, shown in Figure 3e, reveals that MLP deficiency is not
associated with any significant alterations in protein levels of SERCA2a,
PLB and calsequestrin, suggesting that the defects of Ca^{2+} cycling in
15   MLPKO mice is based upon functional impairment of EC coupling, as
opposed to simply reflecting decreases in the protein levels of either
SERCA2a or phospholamban.

         One of the characteristic features of heart failure is the reactivation
of an embryonic gene program which may contribute to a compensatory
20   response to an increased hemodynamic load. To confirm that
hemodynamic improvement in DKO mice is accompanied by amelioration
of changes at the transcriptional level, the expression of ANF, α-skeletal
actin, and β-MHC mRNAs, well established embryonic markers for heart
failure, is examined. As shown in Figures 4a and 4b, ventricles of MLPKO
25   mice display a 26-fold increase in ANF, a 13-fold increase in α-skeletal
actin and an 8-fold enhancement of β-MHC mRNAs. DKO mice exhibits
only a 1.9-fold increase in ANF and no significant increase in α-skeletal
actin or β-MHC mRNAs. Thus, ablation of PLB largely suppresses
induction of the embryonic gene program in MLPKO mice.

30   The aforementioned studies indicate that the ablation of PLB can

rescue independent parameters of heart failure and associated defects in
cardiac contractility. To define the mechanism of the rescue effect, it is
necessary to assess whether the chronic interaction of PLB and SERCA2a
is in fact limiting for cardiac contractility in both normal and myopathic
5   hearts. The "supra-rescue" of basal cardiac function in the DKO mice to
levels comparable to those in wild-type mice following maximal
catecholamine stimulation suggests that inhibition of this interaction may
exert a dominant effect to maximize cardiac performance in the absence
of any catecholamine stimulation.

10

Recombinant Adenoviral transgene mutants of PLB

       Using the knowledge that certain amino acid residues of PLB are
required to maintain its inhibitory effects on SERCA2a, several single point
mutations of PLB, V49A (Seq. ID. No. 2), E2A (Seq. ID. No. 3), R14E
15   (Seq. ID. No. 4), S16N (Seq. ID. No. 5),a double point mutation of PLB,
K3E/R14E (Seq. ID. No. 6) and a sense and antisense PLB (Seq. ID. No. 1)
transgene can be engineered in order to disrupt the inhibitory effects of
PLB on SERCA2a. Using recombinant adenoviruses for *in vivo* murine
cardiac gene transfer, myocytes which overexpresses V49A, one of the
20   single point mutations in PLB, exhibit an increase in contractility, while
myocytes which overexpress the wild-type PLB exhibit a decrease in
contractility when compared to non-infected myocytes, as is documented
in Figure 5. It can be concluded that the feasibility and utility of
interfering with the interaction between the SERCA2a and PLB is clearly
25   documented. The PLB-SERCA2a interaction appears to be the rate
limiting step for establishing the set point of basal cardiac contractility and
relaxation in vivo, and the disruption of this interaction can thereby short
circuit the β-adrenergic pathway.

       Additional Western blot analysis of myocytes containing the
30   adenovirus transgenes expressing sense PLB (sPLB), antisense PLB

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(asPLB), E2A, R14E, S16N, and K3E/R14E against monoclonal PLB antibody (Affinity BioReagents) is shown in Figure 6a. Quantification of PLB protein content, normalized to α-actin for loading variance and compared with an adenovirus/ SR control lacking the transgene, shows

5   that sPLB, E2A, and R14E mutants increase PLB protein level by 150% (PLB_5 + PLB_1), 72 %, and 57%, respectively. In contrast, asPLB and S16N results in 54% and 33% decrease in PLB protein content within the myocytes. The introduction of K3E/R14E transgene infection of myocytes leads to a formation of a distinct pattern of pentamer PLB. Multiple PLB

10   bands appear in addition to PLB_5 (the pentamer). This is accompanied by a reduced abundance of PLB_5 in comparison with the control.

        The nature of the Western blot banding pattern is further evaluated · by substituting PLB-deficient Sol8 cells in place of cardiac myocytes. Sol8 cells are infected with the recombinant adenovirus expressing either

15   the transgene sPLB or K3E/R14E alone or in combination. As seen in Figure 6b, the Western blot shows that the monoclonal PLB antibody detects PLB in cells infected by sPLB but fails to detect K3E/R14E. Infection of Sol8 cells with a combination of the adenoviral transgenes results in formation of multiple bands of PLB. Moreover, the PLB

20   pentamer decreases in abundance simultaneously with the appearance of the upper bands. It is well established that PLB interacts with and inhibits SERCA2a predominantly as a monomer that exists in equilibrium with the noninhibitory pentamer. Based on this knowledge, the heteropentamer of K3E/R14E and wild-type PLB might be more stable that the

25   homopentamer of wild-type PLB. Therefore, the dissociation of the heteropentamer into monomers, which results in inhibition of SERCA2a, is disfavored. K3E/R14E interacts with endogenous PLB and forms such a complex, accompanied by a decrease in homopentamer formation. In as much, the monomer K3E/R14E may act as a noninhibitory competitor for

30   endogenous wild-type PLB by blocking SERCA2a-PLB interaction sites.

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The effects of mutant and antisense PLB on SERCA2a is further
evaluated by determination of the SR calcium uptake activity. The initial
rate of Ca^{2+} uptake by the SR measured at varying Ca^{2+} concentrations
reflects the activity of SERCA2a. As shown in Figure 7, neonatal rat

5   myocytes infected with the recombinant adenovirus transgenes K3E/R14E
and asPLB show a decrease in the concentration of Ca^{2+} needed by
SERCA2a for the same activity compared with the non-transgene control,
indicating a stimulation of SERCA2a activity. The EC_{50} s of Ca^{2+}
concentration at which the uptake activity is half-maximal are, (in μmol/L),

10   0.20 $\pm$ 0.02 for the non-transgene control (SR-), 0.11 $\pm$ 0.01 for
K3E/R14E, and 0.13 $\pm$ 0.01 for asPLB. The effects of K2E/R14E and
asPLB on SERCA2a are also examined in adult rat myocytes. The
adenoviral transgene K3E/R14E lowers the EC_{50} significantly (by 36%),
whereas the change as a result of asPLB infection is not within statistical

15   significance. This apparent discrepancy in the effects between neonatal
and adult cardiac myocytes is possibly related to the different abundances
of PLB in myocytes at different developmental stages. PLB is nearly twice
as abundant in adult as in neonatal myocardium.

To further examine the effects of K3E/R14E and asPLB on

20   SERCA2a, intracellular Ca^{2+} transients in neonatal myocytes are measured
by use of the indo 1 fluorescence indicator. Indo 1 ratiometric data which
is obtained from each of the experimental conditions is normalized to the
respective maximum and minimum of each contractile Ca^{2+} transient and
is then aligned and averaged. As shown in Figure 8, the decay curves of

25   K3E/R14E and asPLB are displaced to the left of the LacZ control.
Furthermore, for most of the diastolic time points, K3E/R14E is
significantly different from LacZ, whereas at several diastolic time points,
asPLB is also significantly different from LacZ. The half-times for decay
(RT_{50}) for LacZ, K3E/R14E, and asPLB are determined to be 0.28 seconds

30   (100%), 0.20 seconds (73%), and 0.22 seconds (79%), respectively.

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The values for K3E/R14E (73%) and asPLB (79%) are significantly different ($p < 0.05$) from the values obtained from the LacZ expressing virus.

5   In addition to generating adenoviral transgenes using various point mutations of PLB, or the sense or antisense sequences of PLB, antibodies raised against PLB peptide and then expressed as RNA can also be inserted into the adenoviral vector. To raise polyclonal PLB antibody ("contractilin", or chicken antibody peptides with hyperactive regions), a chicken is repeatedly immunized with PLB peptide which represents amino

10   acids 3 to19 of the cytoplasmic domain. After three rounds of booster immunization, administered at 15, 42 and 54 days, total IgY is purified from the egg yolk, using a commercially available purification system (EGGstract IgY Purification System - Promega). Upon confirmation of a positive immune response, lymphocytes from the spleen and bone marrow

15   are harvested. RNA, in the form of the hypervariable regions from both antibody light and heavy chain is obtained from these cells and amplified by RT-PCR, the method of which is well known.

       The amplified and purified hypervariable region RNA is then fused to a single cDNA (Seq. ID. No. 9) and subsequently cloned in frame into a

20   plasmid vector, coding for a phage surface protein. Using standard phage display technique, phages which express the immune library of the chicken are selected by their positive response to the PLB peptide. After a series of enrichment for phages which specifically bind PLB, 20 clones are selected for ELISA. The resulting 5 best binders are then analyzed using a

25   radioactive Ca^{2+} transport assay. The two best activators of SR Ca^{2+} transport are further analyzed. Both clones are found to dramatically stimulate the rate of Ca^{2+} transport into the SR.

       To demonstrate that the recombinant protein, which has been generated from contractilin (the PLB antibody), can also function inside a

30   living cell, an adenoviral vector expressing contractilin is constructed.

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Western blot analysis of neonatal and adult rat cardiomyocytes, infected with the adenoviral transgene, shows that contractilin can be expressed in heart cells. Radioactive Ca^{2+} transport analysis indicate that, as with the mutant and antisense PLB, contractilin accelerates cytoplasmic Ca^{2+}

5   removal.

In a complementary approach, plasmid transfection rather than adenoviral transfection is used for gene delivery. It is found that K3E/R14E- and asPLB- transfected myocytes, as monitored by co-transfected green fluorescence protein, exhibits 43% ($p < 0.05$) and 9%

10   ($p < 0.1$) decreases in RT_{50}, respectively, relative to adenoviral vector transfected cells. Thus, introducing K3E/R14E and asPLB into the cardiac myocytes by either the adenovirus or co-transfection technique reduces the duration of the diastolic Ca^{2+} transients. These results would seem to mirror the findings of MLPKO versus DKO mice where variation in Ca^{2+}

15   transients confirm that ablation of PLB is associated with a shortened duration of Ca^{2+} transient, faster decay, and preserved amplitude. Taken together, these data confirm that K3E/R14E and asPLB stimulate the SERCA2a activity, which results if faster Ca^{2+} transients in myocytes.

To determine whether the enhanced SERCA2a activity and

20   accelerated Ca^{2+} transients, as a result of the PLB mutants, lead to change in contractile behavior, edge detection is used to analyze myocyte contractility. Adult rabbit myocytes are infected with the adenoviral transgenes of LacZ, K3E/R14E, or asPLB. After a three day incubation period, there is a significant difference in the number of spontaneously

25   contracting cells between the different groups (LacZ < < asPLB < K3E/R14E). Table 2 provides the effects of K3E/R14E and asPLB on myocyte contractility. As shown in the table, compared with the LacZ control, K3E/R14E increases fractional shortening by 74%, which is accompanied by a 25% decrease in RT_{50} and a 115% increase in $+dL/dt$.

30   When the myocyte contractility is examined after asPLB infection, it is

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found that the fractional shortening of the myocytes increases significantly, by 57%, whereas the changes in RT_{50} and +dL/dt are not significant.

Table 2

	LacZ (n = 33)	K3E/R14E (n = 29)	asPLB (n = 30)
+dL/dt (μm/s)	11.7 ± 1.9	25.1 ± 1.6*	18.4 ± 2.0*
RT_{50} (ms)	539.0 ± 27.0	402.0 ± 19.0*	483.0 ± 29.0***
Shortening (%)	6.2 ± 0.5	10.8 ± 0.5*	8.0 ± 0.6***

*: $p < 0.05$
**: $p < 0.1$

The resulting data show that the increase in SERCA2a activity translates into an accelerated relaxation of the myocytes. K3E/R14E-infected myocytes display an enhanced fractional shortening, which suggests an increase in SR loads of Ca^{2+} due to the enhancement of SERCA2a activity. Further, K3E/R14E infection increases the number of spontaneously contracting myocytes, a phenomenon most likely associated with the increased amount of oscillating Ca^{2+} due to the elevated SR loading of Ca^{2+}. Taken together, these data show that K3E/R14E affects endogenous wild-type PLB in a way that significantly reduces its inhibition of SERCA2a and thus has a dominant inhibitory effect over wild-type PLB.

Peptide-based therapeutic for inhibition of PLB activity

Still further, the present invention provides for a peptide based therapeutic for the inhibition of phospholamban activity and a mode of delivery for such a therapeutic, based on the finding that PLB function can be inhibited in a dominant negative manner by overwhelming endogenous PLB with mutant PLB molecules, and that this inhibition leads to improved function in failing hearts.

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For a therapeutic agent, such as an inhibitor of the PLB-SERCA2a interaction, to effect a target cell system, it must have a means for internalization through the cell membrane into the cytoplasm. The mode of transfer of the inhibitor can be by way of either a transport or

5   penetratin based PLB peptide or it can also include adenoviral or lipid vesicle based transfer. For this purpose, a compound consisting of a fusion of a transport peptide and a PLB protein molecule is constructed. The transport peptide comprises a 16-residue from the sequence for antennapedia, a Drosophila transcription factor protein. The second

10   peptide of the complex can be a truncated sequence of PLB protein. Further therapeutic benefits can be achieved using peptides that correspond to native PLB protein as well as a mutant or truncated form of PLB protein.

One beneficial function of the transport peptide-PLB complex is the

15   inhibition of the interaction between PLB and SERCA2a within cardiomyocytes, resulting in enhanced contractility in diseased hearts. The present invention may also inhibit the interaction of PLB with SERCA2a within the smooth muscle layer surrounding the arteries/ arterioles of the circulatory system which would result in vasodilation and

20   reduced blood pressure. Thus, there is a two-fold benefit in the treatment of heart disease, the first is enhanced cardiac contractility in failing hearts, the other is the reduction of blood pressure in individuals with hypertension. It is also predicted that there will be the inhibition of PLB interactions with SERCA proteins of other cell types, such as the

25   SERCA1-PLB interaction in nervous tissue.

The introduction of the molecule into the blood stream feeding the heart can is best achieved using a catheter located in the coronary artery. When the molecule is present in the extracellular environment surrounding a cardiomyocyte it rapidly enters the cardiomyocyte and inhibits the

30   association of PLB with SERCA2a. The translocation function is

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attributable to the transport peptide which exhibits the ability to rapidly translocate itself and the attached "cargo" peptides across the cell membranes in a receptor independent manner. Once inside the cytoplasm of the cardiomyocyte, the PLB fragment will act as a competitive inhibitor

5   of endogenous PLB interactions with SERCA2a.

In the absence of PLB inhibition by association, SERCA2a more efficiently pumps Ca 2+ into the SR, thereby increasing the cardiomyocytes ability to contract more strongly and rapidly. Stronger cardiomyocyte contractility translates to more powerful heart contractility.

10   *In vivo*, the present invention could act as a treatment for heart failure and is most easily administered and most effective in patients whose hearts require, or already have implanted, a left-ventricular assist device (LVAD).

While residues 43 to 58 of Antennapedia is a well characterized translocation peptide, and works well in the present invention, the present

15   invention is not restricted to this method of transport. Other potential methods of transfer include the use of an 8-branched polylysine backbone to link the transport and cargo peptide, but it is not limited to this multi-branched structure. A compound consisting of one target peptide attached to one PLB peptide, as one long peptide, has also been explored.

20   Still further, a number of DNA constructs for producing hexahistidine (H6) tagged penetratin and penetratin-PLB recombinant proteins in bacteria have been undertaken. The penetratin peptides were engineered to be on either the amino or carboxy terminal end of the protein.

It has been shown that the cytoplasmic fragment of PLB has as

25   strong a binding affinity for the cytoplasmic portion of SERCA2a as the whole PLB molecule. Therefore, once the transport-PLB molecule is inside the cytoplasm of the cardiomyocyte, the PLB fragment is predicted to act as a competitive inhibitor of endogenous PLB interaction with SERCA2a.

This form of treatment is suitable for the patient who is suffering

30   from severe decreased cardiac pump function, refractory to medical

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therapy, and requiring mechanical assistant devices while waiting for heart transplantation. In addition, the underlined molecular mechanisms for the dominant negative function of the PLB mutants can be used in the design and implementation of the high-throughput screening strategies for

5   inhibitory small molecules.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE 1

10   Creation of knock-out mouse lines for echocardiography

In order to analyze the structural and physiological features of the complex *in vivo* heart failure phenotype of dilated cardiomyopathy, several lines of knock-out mice were created. conducted using a double knock-out (DKO) mouse model which harbors homozygous ablation of two

15   independent muscle specific genes. For this strategy, PLB $^{-/-}$ (phospholamban deficient) homozygous mice were mated with MLP $^{-/-}$ (muscle- specific LIM protein) homozygous mice. The F1 pups generated from an MLP$^{-/-}$ x PLB$^{-/-}$ homozygote cross were then mated to create the MLP$^{+/-}$, PLB$^{+/-}$ double heterozygote genotype. F2 offspring were

20   generated from a MLP$^{+/-}$ / PLB$^{+/-}$ double heterozygote mating, thereby creating mice that were homozygous for the mutant MLP allele and heterozygous for the mutant PLB allele or that were MLP wild-type and heterozygous for the mutant PLB allele. The F3 offspring were generated from a MLP$^{-/-}$ / PLB$^{+/-}$ matings to generate MLP$^{-/-}$ / PLB$^{-/-}$ (DKO), MLP$^{-/-}$ /

25   PLB$^{+/+}$ (MLPKO) and MLP$^{+/+}$ / PLB$^{-/-}$ (MLPKO/ PLBhet) littermates or from a MLP$^{+/+}$ / PLB$^{+/-}$ matings to generate MLP$^{+/+}$ / PLB$^{-/-}$ (PLBKO), MLP$^{+/+}$ / PLB$^{+/+}$ (wild-type) and MLP$^{+/+}$ / PLB$^{+/-}$ (PLBhet) littermates. The genotype of the gene-targeted crosses were determined by PCR or genomic DNA isolated from tail biopsies.

30   To evaluate the hemodynamic properties of the various knock-out

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mouse lines, cardiac catherization and echocardiography was performed
on subjects anesthetized with either Avertin (2.5%, 20 μl/kg body weight)
or xylazine (0.005 mg/g) and ketamine (0.1 mg/g). Transthoracic M-mode
echocardiographic tracings indicated that MLPKO mice had chamber

5   dilation with reduced wall motion, indicating depressed cardiac function
and increased wall stress, whereas chamber size and cardiac function are
normal in the DKO mice. Baseline parameters for wild-type (WT), n = 7,
MLPKO, n = 8, DKO, n = 9, and PLBKO, n = 5 are shown in Figures 2a-d.
Data were expressed as mean ± SEM. MLPKO versus other groups;

10   *$p < 0.5$, **$p < 0.001$, WT vs. DKO; #$p < 0.01$. In Figures2e-h,
hemodynamic assessment was made of β-adrenergic responsiveness to
progressive infusion of dobutamine, where WT ($\square$), n = 7, MLPKO ($\bullet$),
n = 8, and DKO ($\bigcirc$), n-9, mice. MLPKO vs WT or DKO; #$p < 0.05$,
+$p < 0.01$, *$p < 0.001$, WT vs DKO; ∋$p < 0.01$.

15

EXAMPLE 2

Calcium transient analysis

         To evaluate the effect of inhibition of PLB on SR calcium content
and calcium transients, myocytes were isolated from the right ventricular

20   wall of the wild-type or knock-out mice. To monitor the changes in
intracellular calcium, the isolated myocytes were incubated with a calcium
sensitive dye, fluo-3-AM (1 μg/ml), for 30 minutes at room temperature.
The myocytes were then transferred to a tissue chamber on the stage of
an inverted microscope and continuously stimulated at a rate of 1 Hz to

25   maintain a consistent degree of SR calcium loading. To measure cellular
fluorescence, the myocytes were illuminated with an excitation
wavelength of 480 nm. Any changes in fluorescence were monitored at
510 nm using a microfluorometer (FM-1000; Solamere Technologies) and
digitally recorded for later analysis using Cellsoft (D. Bergman; University

30   of Calgary) software. Fluorescence values were calibrated using the

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equation:

$$[Ca^{2+}]i = K_D(F\text{-}Fmin)/(Fmax\text{-}F) \hspace{2cm} (1)$$

with an assumed K_D of 864 nM, where F are the experimentally derived fluorescence values. Fmax was determined by adding 10 μM ionomycin

5   to the superfusion solution and Fmin was determined by adding 4 mM $MnCl_2$ to the superfusion solution for each myocyte.

SR calcium content of the isolated myocytes was assessed using a standard caffeine pulse protocol. Following stable recordings of calcium transients, a 20 second pulse of 10 mM caffeine was applied to the

10   myocyte. This protocol resulted in a rapid caffeine-induced transient which slowly decayed back to baseline values. The SR calcium content was defined as the integrated area of this caffeine-induced calcium transient. Figure 3a illustrates the representative intracellular calcium transient in the WT, MLPKO and DKO myocytes. MLPKO myocytes

15   exhibited an attenuated amplitude of calcium transients with normal levels of diastolic calcium concentration. DKO myocytes displayed the calcium transient with a shortened duration, faster decay, and preserved amplitude. As shown in Figure 3b, the amplitude of calcium transient was significantly attenuated in MLPKO myocytes and was restored in DKO

20   myocytes. Figure 3c shows that intracellular diastolic calcium concentration was not different among the three different groups of myocytes. In Figure 3d, SR calcium content was significantly decreased in MLPKO mice and increased in DKO mice when compared to WT mice. In Figure 3e, representative quantitative immunoblotting revealed that MLP

25   deficiency was not associated with any significant alterations in the protein levels of SERCA2a, PLB, and calsequestrin.

EXAMPLE 3

Construction of mutant PLB adenovirus and gene transfer

30   I.M.A.G.E. consortium cDNA clones encoding human PLB were

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available through Genome System, Inc. The DNA fragment harboring the
entire coding sequence of PLB was subcloned into pBluescriptII KS vector,
as well known E. coli cloning vector (ATCC accession no. 87047). A
sense mutation (Val49Ala) was introduced using a PCR based

5   mutagenesis system commercially available from Stratagene.
Recombinant adenovirus expressing wild-type and mutant human PLB was
generated by homologous recombination between plasmid pJM17 and a
shuttle plasmid containing RSV promoter and SV40 poly A sequences.
The concentrated virus preparation were tittered using a standard

10   protocol. The efficient *in vivo* cardiac gene transfer was performed by
injecting the adenovirus vectors into 1 day old neonatal mouse heart. The
myocytes were isolated 4 weeks after injection into the mouse hearts and
cell shortening was measured. Myocytes harboring the mutant transgenes
were identified by co-transfection of adenoviral vectors expressing GFP as

15   a marker.

EXAMPLE 4

Construction of a PLB inhibitor-transport peptide complex

A PLB inhibitor molecule was made by indirectly attaching a

20   transport peptide and a PLB protein to a polylysine backbone.
Alternatively, the PLB molecule could also have been made as a single
long peptide consisting of a transport sequence tandemly attached to the
cargo peptide sequence. The transport peptide was composed of residues
43 to 58 of antennapedia (Seq. ID. No. 7), a Drosophila transcription

25   factor protein. The cargo peptide was derived using the first 16 residues
of PLB (Seq. ID. No. 8). It is important to note that this cargo sequence
could also have been derived from any segment of wild-type PLB or
mutant PLB.

The PLB inhibitor molecule was constructed by linking 4 transport

30   peptides with 4 peptides matching the first 16 residues of PLB. The

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backbone linker was an 8-branch lysine, commonly used in multiple antigenic peptide (MAP) synthesis. The first 4 branches of the MAP resin were used to synthesize the antennapedia peptide. The next 4 branches were then deprotected and used as the starting point for the synthesis of

5   the PLB cargo peptide. Thus, this particular PLB inhibitor that was used for initial characterization had 4 branches of the antennapedia peptide and 4 branches of the PLB cargo peptide. Alternatively, the PLB inhibitor could have been constructed as a single peptide with the cargo and transport peptides attached to each other by a single peptide bond, or as

10   the cargo and transport peptides attached to each other by a disulfide bond. The PLB inhibitor molecule was translocated efficiently into isolated neonatal rat cardiomyocytes and showed a resulting enhanced contractility of the cell, the results of which can be seen in Figures 5a and b. Myocytes that overexpressed the V49A PLB point mutation showed

15   increased contractility, while myocytes which overexpressed the wild-type PLB exhibited decreased contractility when compared to non-infected myocytes.

EXAMPLE 5

20   Penetratin peptides TAT and ANT

Cell level studies were done to evaluate the ability of two penetratin -based peptides, two mutant PLB-penetratin peptides, and two multiple antigen peptides (MAP) to strengthen the contraction cycle of isolated mouse cardiomyocytes. The two penetratin-based peptides include PLB-

25   ANT (Seq. ID. No. 10) and TAT-PLB (Seq. ID. No. 11) each of which have a 20 residue portion of the PLB sequence attached to either the 5' end of ANT (Seq. ID. No. 14) or the 3' end of TAT (Seq. ID. No.15). The two mutant PLB peptides, mutant PLB-ANT (Seq. ID. No. 12) and TAT-mutant PLB (Seq. ID No. 13), display a S16E mutation of the 20 residue PLB

30   sequence. The multiple antigen peptides include MAP with 8 penetratin

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(ANT) domains and MAP with 4 penetratin domains and 4 PLB domains .

Each of the penetratin-PLB peptides were evaluated to measure their ability to strengthen the contraction cycle of isolated mouse cardiomyocytes, the idea being that the penetratin-PLB peptide would act as a dominant negative inhibitor of the PLB-SERCA2a interaction. The results of the TAT-PLB peptide on the isolated cardiomyocytes is shown in Table 3. For this data, tests were repeated on several sets of cardiomyocytes to determine relative change in length through the contraction cycle with the TAT-PLB peptide (samples 1-7) and without the peptide (controls 1-8). Each of the samples had an added concentration of 10 μM of the TAT-PLB peptide while the controls had no added peptide.

Table 3

	Maximum Contract.	Minimum Contract.	% Contract.	Δ Length/ msec.	r2 (fit)	Contract. Slope	Relax. Slope
control 1	100.63	92.978	7.604	-34.503	0.9696	29.935	0.9955
control 2	91.146	83.301	8.607	-38.76	0.9943	28.736	0.9349
control 3	115.45	100.05	13.339	-82.875	0.9768	89.054	0.9894
control 4	105.00	102.04	2.819	-19.196	0.9842	10.497	0.9944
control 5	83.747	79.637	4.908	-21.695	0.9971	12.184	0.9742
control 6	145.96	136.85	6.241	-50.185	0.9721	27.566	0.9912
control 7	154.56	142.16	8.023	-76.607	0.9933	73.70	0.9928
control 8	115.59	102.68	11.169	-59.643	0.9765	63.304	0.9789
Mean	**114.010**	**104.962**	**7.839**	**-47.933**	**0.9830**	**41.872**	**0.9814**
sample 1	112.57	101.17	10.127	-65.554	0.9865	59.875	0.9925
sample 2	109.68	102.00	7.002	-30.267	0.9609	37.157	0.9790
sample 3	133.82	116.32	13.077	-79.242	0.9964	134.46	0.9878
sample 4	81.61	67.871	16.835	-58.093	0.9961	65.017	0.9697
sample 5	74.423	64.629	13.160	-54.353	0.9539	47.775	0.9933
sample 6	126.89	108.38	14.587	-98.054	0.9819	107.07	0.9939
sample 7	133.61	128.21	4.042	-36.071	0.9966	27.911	0.9959
Mean	**110.373**	**98.363**	**11.261**	**-60.233**	**0.9818**	**68.466**	**0.9874**

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Measurements were taken every 20 milliseconds, and the unit of length
was arbitrary, but was generally on the order of one complete cell length.
Percent contraction was calculated as (maximum length minus minimum
length) divided by maximum length. A plot of time versus length

5   generated a U-shaped curve from which the most linear segments were
selected, with the left side of the "U" representing contraction and the
right side representing relaxation. The r2 column shows how well the data
fit the curve, where1.0 represents a perfect fit.

While there appeared to be a trend towards a larger, faster

10   contraction in the myocyte, T-test analysis not identify any statistical
difference due to the high variability of the data.

EXAMPLE 6

Hexahistidine tagged penetratin

15   A number of DNA constructs were generated for producing
hexahistidine (H6) tagged penetratin and penetratin-PLB recombinant
proteins in bacteria. Using a commercially available expression vector,
pRSET (Invitrogen), recombinant protein H6-ANT (Seq. ID. No16) was
generated. While this recombinant protein has no PLB attached, it was

20   engineered to have epitope tags which was used to detect the protein as
it entered the heart. A variation of H6-ANT was also expresses containing
the PLB sequence, H6-wtPLB-ANT (Seq. ID. No. 17), in addition to an H6-
PLB(S16E mutant)-ANT protein and an H6-PLB (V49A mutant)-ANT
protein (Seq. ID. Nos. 18 and 19 respectively). H6-beta-galactosidase-

25   ANT, H6-TAT, and H6-beta-galactosidase-TAT were also expressed at
lower levels (seq. not listed). A non-functional ANT-penetratin with two
mutations at residues 68 and 67, where Trp was mutated to Phe were
made as negative control for the other three penetratin-PLB proteins.

To measure the effect these recombinant penetratin-based proteins

30   have on cardiac contraction, one mouse was injected intraperitoneally

-32-

with 2 mg of the H6-ANT peptide. A second mouse was injected intraperitoneally with 2 mg of the H6-ANT mutant protein. After a 3 hour incubation period, the mice were sacrificed and the hearts removed for analysis. The blood in the heart was removed by forcing fluid backwards

5   through the aortic arch. Each heart was dissected into atrial tissue, left ventricular tissue, and right ventricular tissue. All the tissue was washed extensively in a physiologically balanced PBS solution and flash frozen in liquid nitrogen. The tissue were then lysed in 8 M Urea, 2% triton-X100, for 10 minutes and equal amounts of the supernatants were

10   electrophoresed on 15% PAGE. The bands were transferred to a PVDF membrane. The membranes were labeled with anti-His antibody in order to identify if the lysate contained the epitope tagged protein.

The invention disclosed herein provides several methods for the treatment of heart failure through the inhibition or alteration of the

15   interaction between phospholamban and sarcoplasmic reticulum Ca^{2+} - ATPase within the cardiomyocytes. Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only

20   by the following claims.

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What is claimed is:

1.   A method for treatment of heart failure comprising inducing phospholamban deficiency.

2.   The method for treatment of heart failure of claim 1, wherein

5   an exogenous phospholamban protein induces phospholamban deficiency.

3.   The method for treatment of heart failure of claim 2, wherein the exogenous phospholamban protein is selected from the group consisting of mutations of PLB, sense PLB, antisense PLB, truncated PLB, native PLB, and antibody against PLB.

10   4.   The method for treatment of heart failure of claim 3, wherein the mutations of PLB comprise point mutations of PLB.

5.   The method for treatment of heart failure of claim 3, wherein the antibody against PLB comprises contractilin.

6.   A peptide based therapeutic agent for inhibiting

15   phospholamban activity consisting of a first peptide and a second peptide as a complex, wherein the first peptide comprises a transport peptide and the second peptide comprises a cargo peptide.

7.   The peptide based therapeutic agent of claim 6, wherein the transport peptide is selected from the group consisting of penetratin,

20   adenovirus , bacterial and lipid vesicle based transport peptide.

8.   The peptide based therapeutic agent of claim 6, wherein the cargo peptide is selected from the group consisting of mutations of PLB, sense PLB, antisense PLB, truncated PLB, and native PLB protein.

9.   The peptide based therapeutic of claim 6, wherein the first

25   peptide transports the second peptide across a cell membrane.

10.   The peptide based therapeutic of claim 6, wherein the first peptide and the second peptide are linked by a covalent linkage.

11.   The peptide based therapeutic of claim 10, wherein the covalent linkage consists of a branched polylysine backbone, a single

30   peptide bond, or a disulfide bond.

12. A method for treatment of heart failure comprising enhancement of cardiac contractility by inhibition of PLB-SERCA2a interaction.

13. The method of claim 12, wherein the cardiac contractility is enhanced by inhibiting effect of PLB on sarcoplasmic reticulum Ca^{2+} ATPase.

14. The method of claim 12, wherein an exogenous phospholamban protein is used to inhibit phospholamban deficiency.

15. The method of claim 14, wherein the exogenous phospholamban protein is selected from the group consisting of mutations of PLB, sense PLB, antisense PLB, truncated PLB, native PLB, and antibody against PLB.

16. The method of claim 15, wherein the mutations of PLB comprise point mutations of PLB.

17. The method for treatment of heart failure of claim 15, wherein the antibody against PLB comprises contractilin.

Fig. 1

Fig. 2

Fig. 2e

Fig. 2f

Dobutamine (μg/kg/min)

Fig. 2g


Fig. 2h

basal 0.75   2   4
Dobutamine (μg/kg/min)
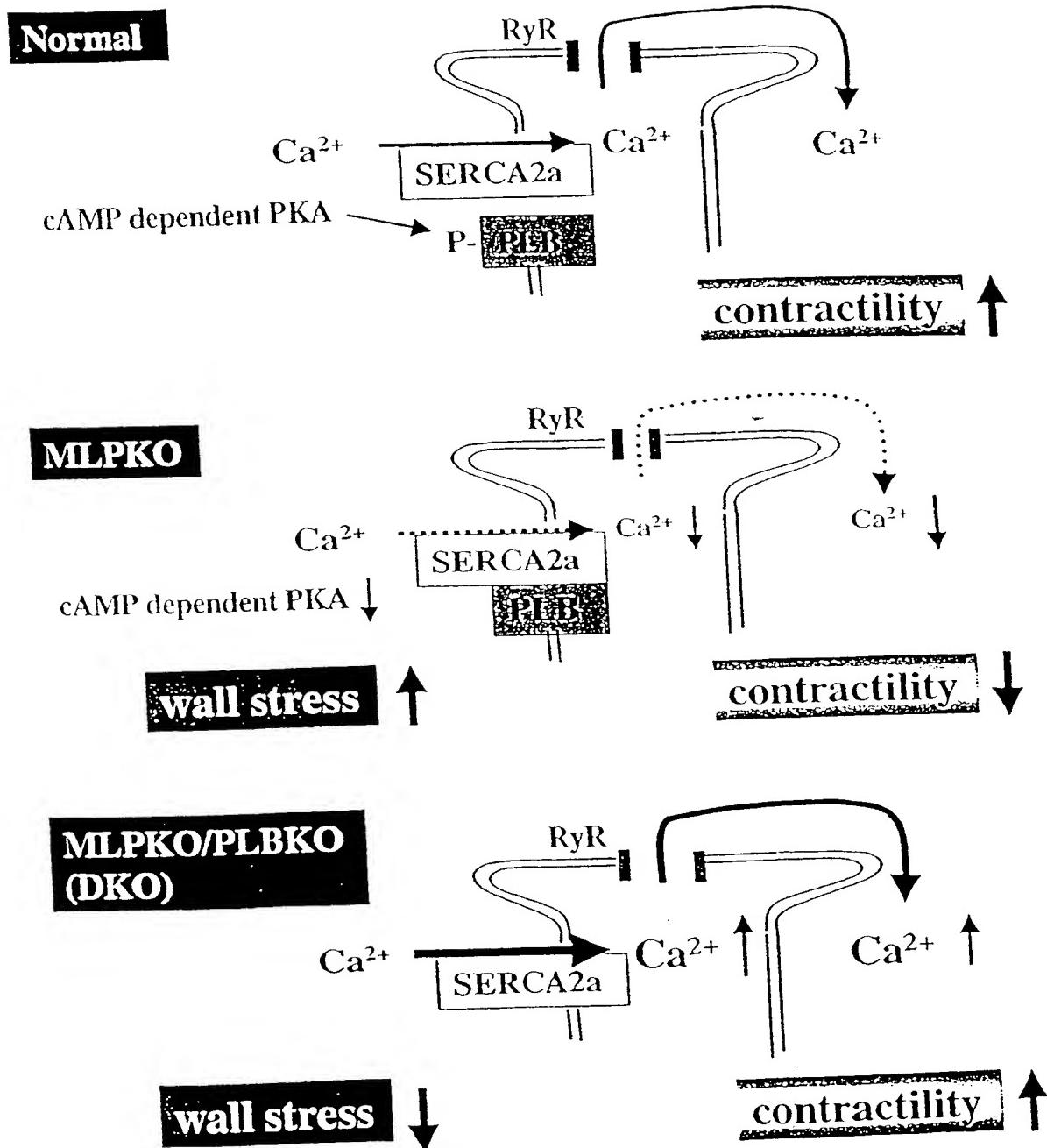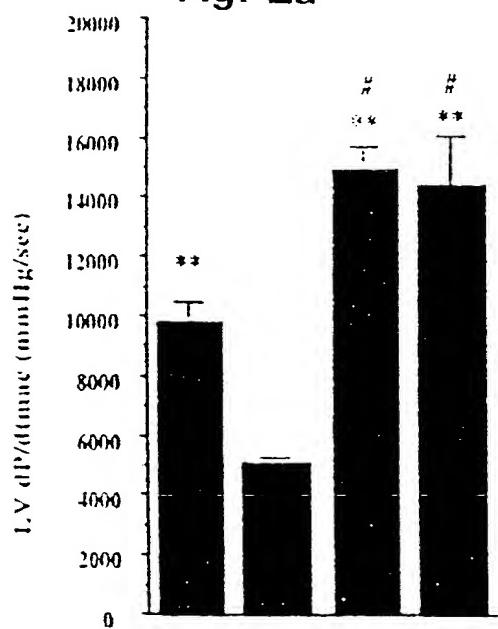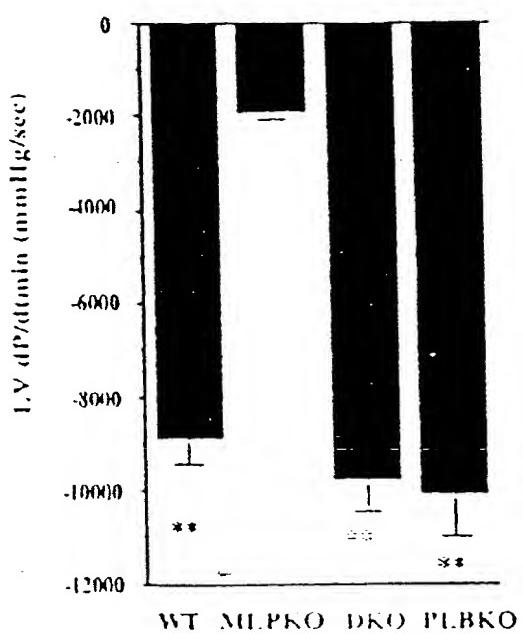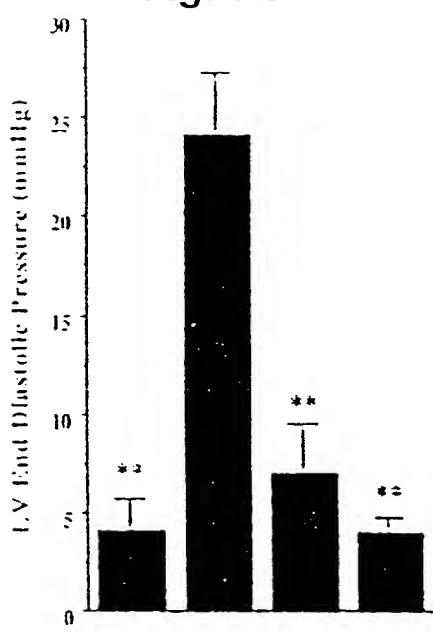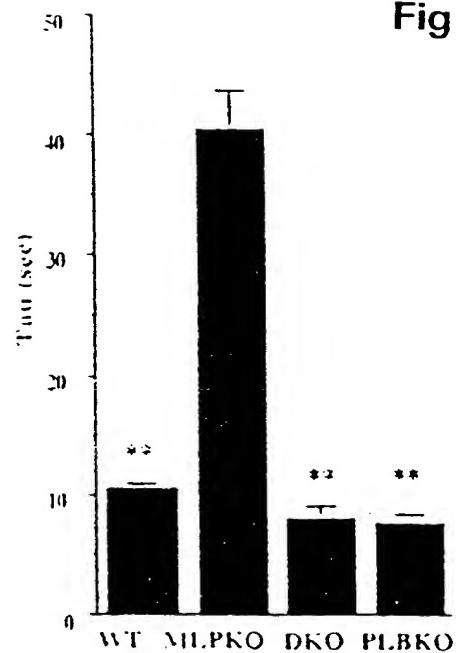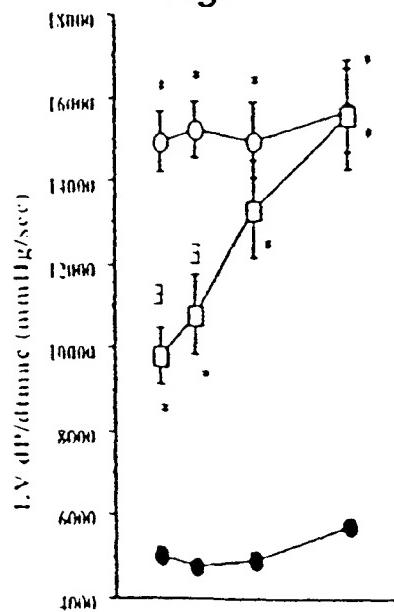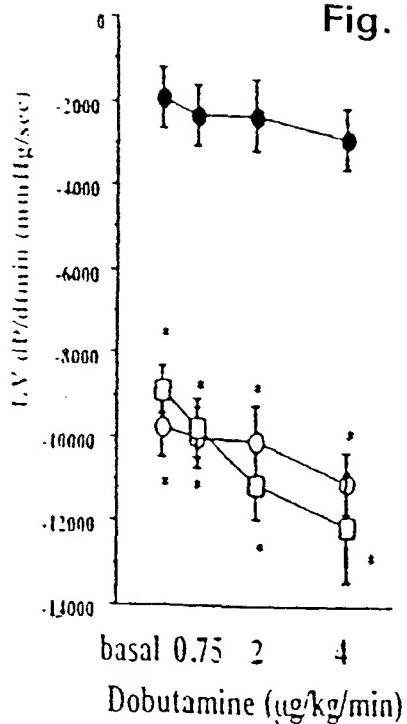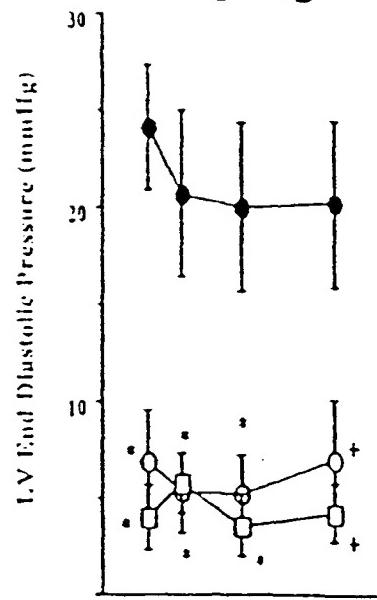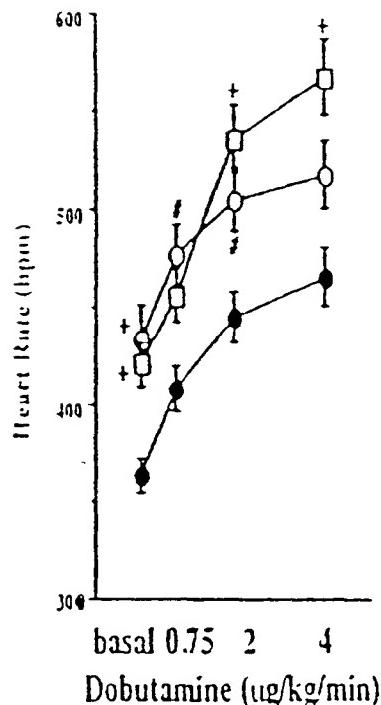
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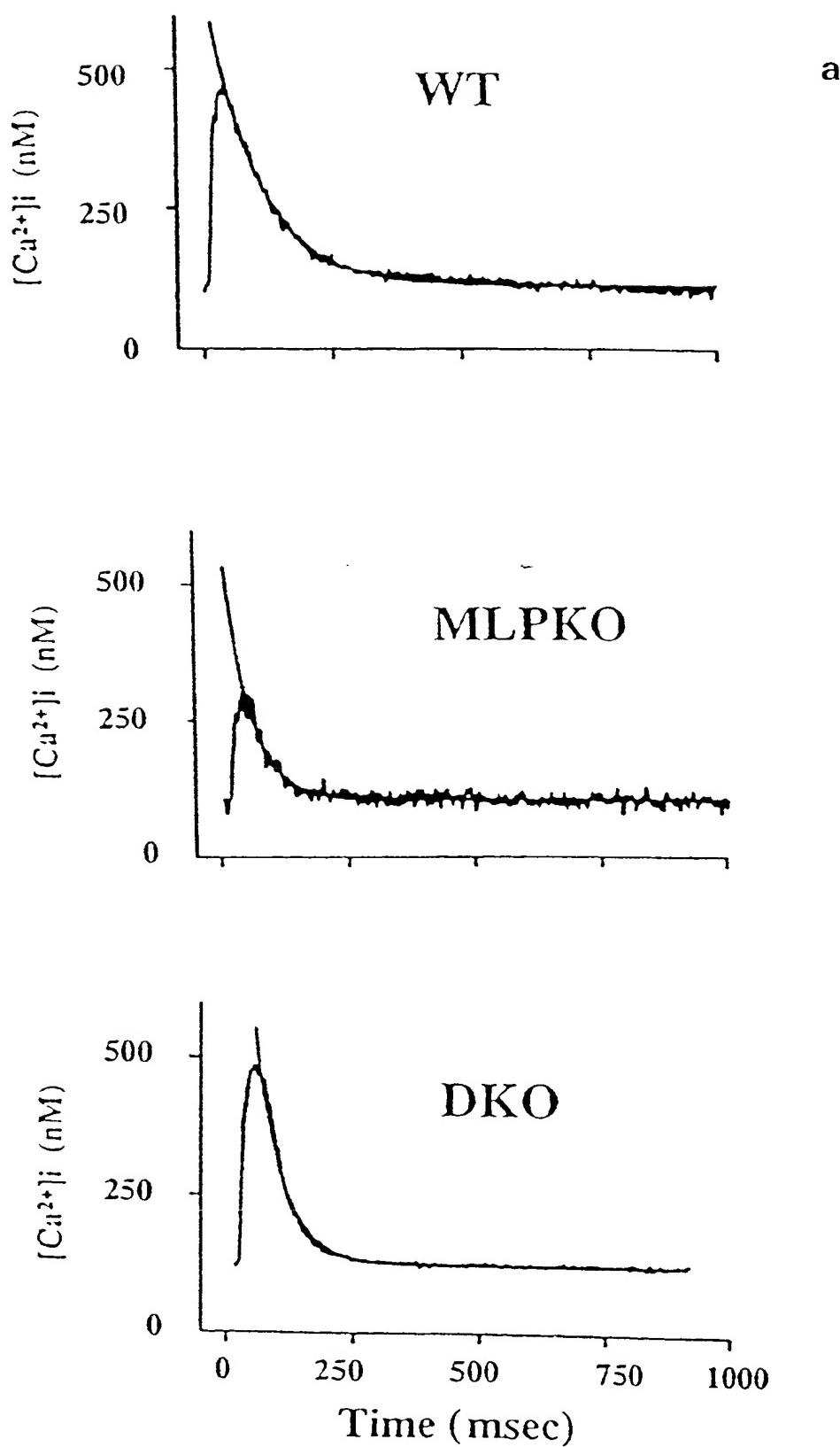
Fig. 3a

Fig. 3b
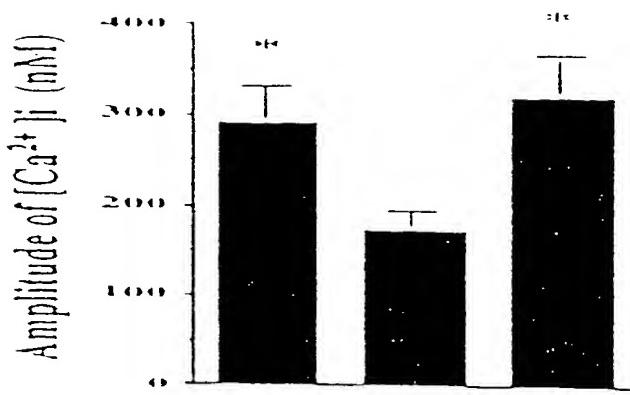
Fig. 3c

Fig. 3d

Fig. 3

Fig. 3 e

Fig. 4a

b

Fig. 4b

Fig. 5

Fig. 5a
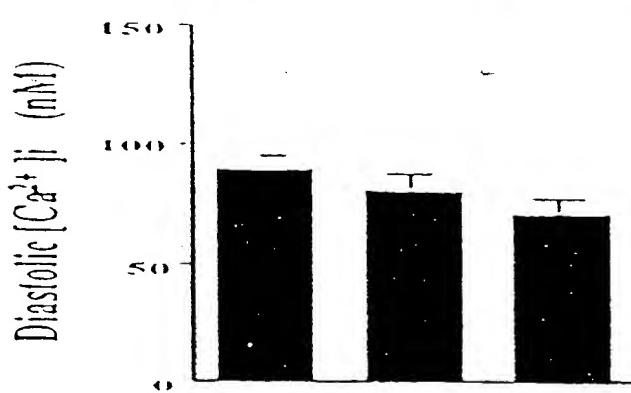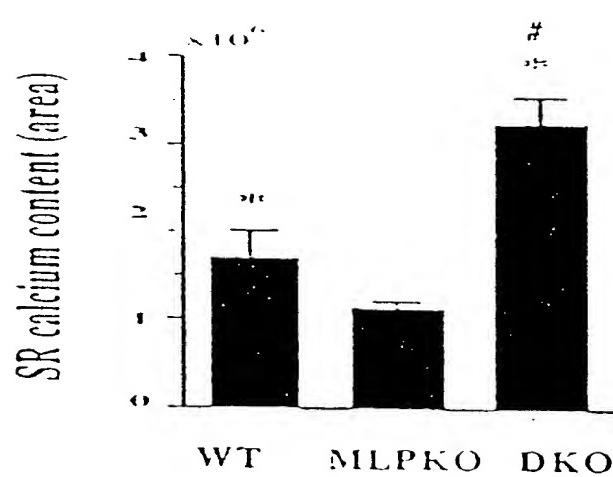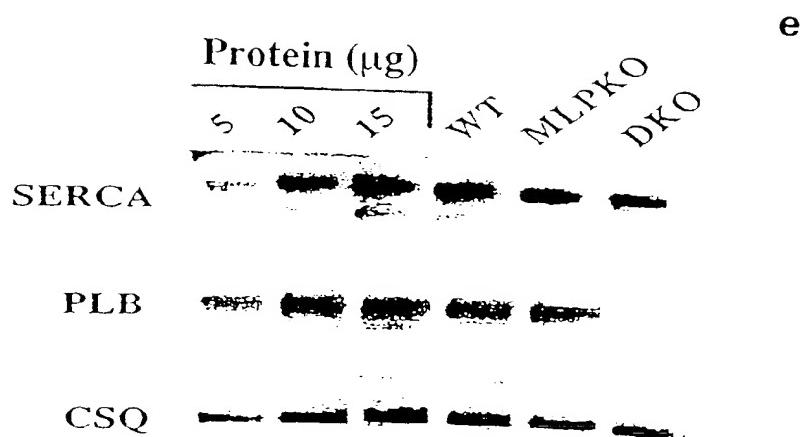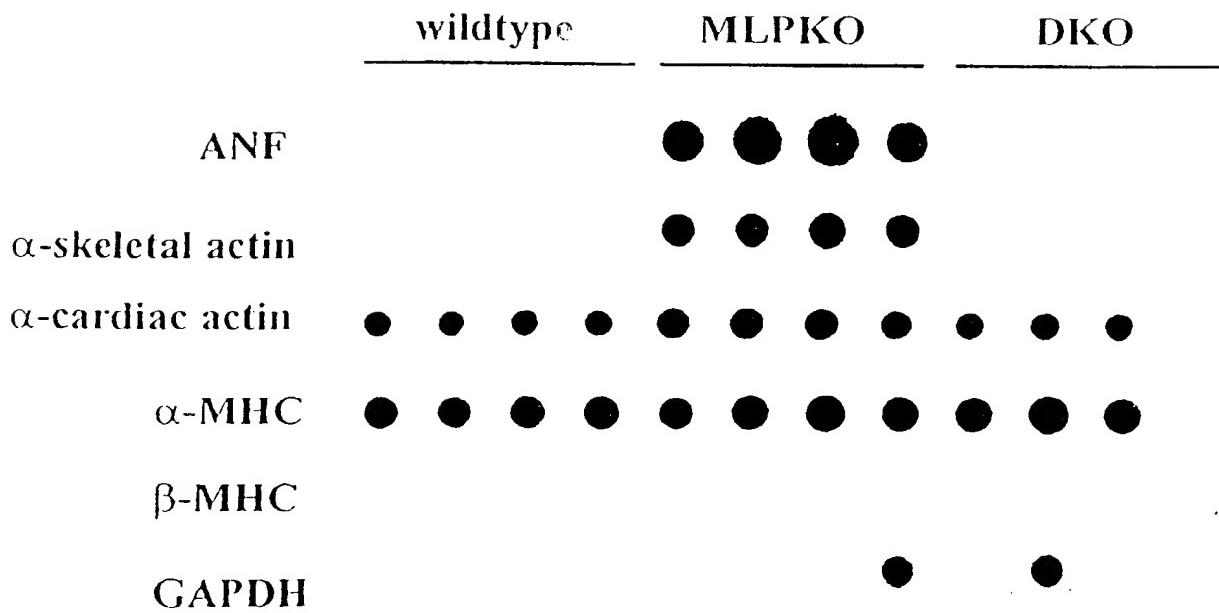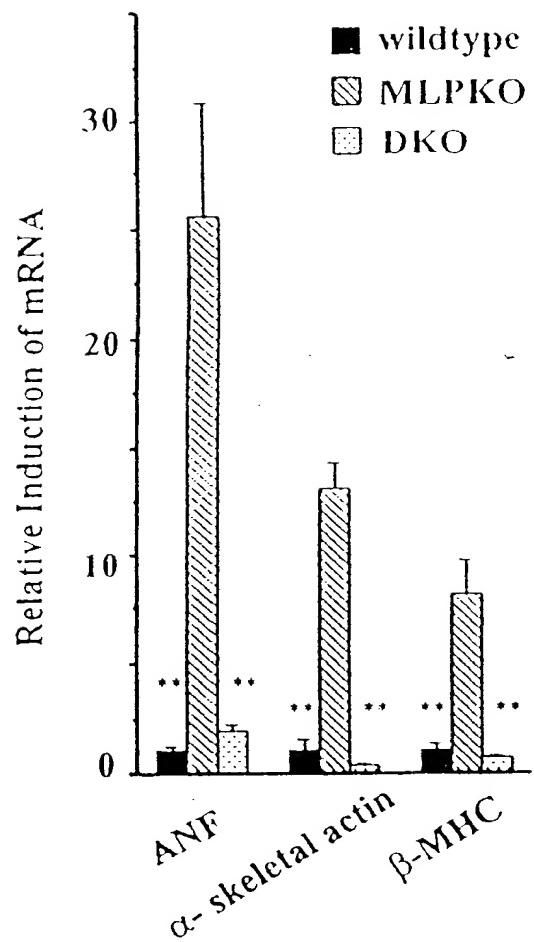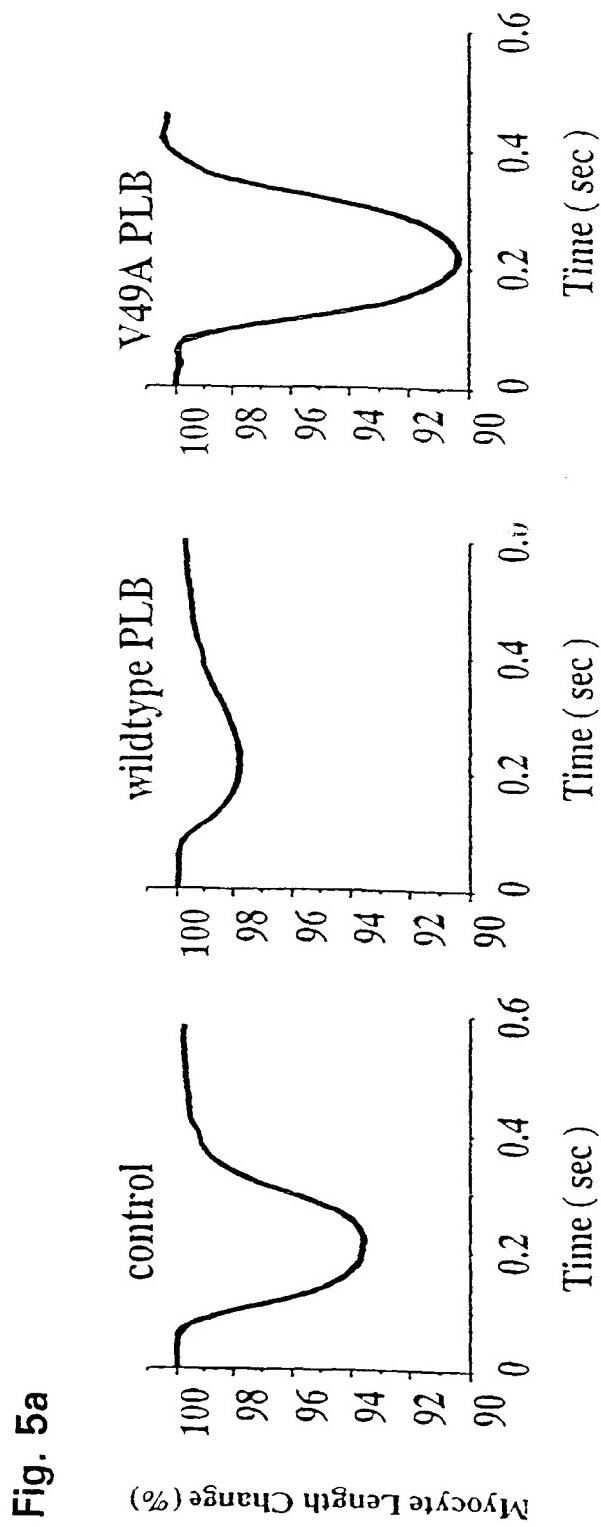
Fig. 5b

	control n=7	wildtype PLB n=3	V49A mutant PLB n=4
Shortening (%)	7.20 ± 0.60	3.50 ± 0.24 *	11.45 ± 0.88 **
-dL/dt (mm)	88.4 ± 10.3	48.4 ± 5.9 *	150.3 ± 9.6 **†
+dL/dt (mm)	56.6 ± 6.5	28.1 ± 6.4 †	116.5 ± 13.5 **†
Cell length (µm)	104.5 ± 3.1	109.2 ± 3.9	108.7 ± 3.3

Fig. 6a

Fig. 6b

Fig. 6c

Fig. 6

Fig. 7

Fig. 8

SEQUENCE LISTING

<110>   Kenneth Chien, Wolfgang Dillman, Susumu Minamisawa, Huaping He, Masahiko Hoshijima, Markus Meyer, Christopher Scott, Yibin Wang, Gregg Silverman

<120>   A METHOD FOR INHIBITION OF PHOSPHOLAMBAN ACTIVITY FOR THE TREATMENT OF CARDIAC DISEASE AND HEART FAILURE

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<141>   November 2, 1999

<150>   US 60/106,718

<151>   November 2, 1998

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      20   25   30   35

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      20   25   30   35

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1   5   10   15

Glu Met Pro Gln Gln Ala Arg Gln Lys Leu Gln Asn Leu Phe Ile Asn Phe Cys
      20   25   30   35

Leu Ile Leu Ile Cys Leu Leu Leu Ile Cys Ile Ile Val Met Leu Leu   52
          40   45   50

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<213>   Human Phospholamban

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 1   5   10   15

Glu Met Pro Gln Gln Ala Arg Gln Lys Leu Gln Asn Leu Phe Ile Asn Phe Cys
    20   25   30   35

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Glu Met Pro Gln Gln Ala Arg Gln Lys Leu Gln Asn Leu Phe Ile Asn Phe Cys
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Tyr Ala Gly Ser Tyr Tyr Tyr Gly Trp Phe Gln Gln Lys Ser Pro Gly Ser Ala

                    40   45   50

Pro Val Thr Val Ile Tyr Ser Asn Asp Gln Arg Pro Ser Asn Ile Pro Ser Arg
55   60   65   70

Phe Ser Gly Ser Thr Ser Gly Ser Thr Ser Thr Leu Thr IleThr Gly Val Arg
          75   80   85   90

Ala Glu Asp Glu Ala Val Tyr Phe Cys Gly Ser Asn Ser Gly Thr Gly Tyr Val
                95   100   105

Gly Ile Phe Gly Ala Gly Thr Thr Leu Thr Val Leu Gly Gln Ser Ser Arg Ser
          110   115   120   125

Ser Thr Val Thr Leu Asp Glu Ser Gly Gly Gly Leu Gln Thr Pro Gly Gly Ala
                130   135   140

Leu Ser Leu Val Cys Arg Ala Ser Gly Phe Thr Phe Ser Arg Phe His Met
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Met Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asp
                165   170   175

Asp Gly Gly Ser Phe Thr Leu Tyr Gly Ala Ala Val Lys Gly Arg Ala Thr Ile
180   185   190   195

Leu Arg Asp Asn Gly Gln Ser Thr Val Arg Leu Gln Leu Asp Asn Leu Arg
          200   205   210

Pro Glu Asp Thr Ala Thr Tyr Phe Cys Val Lys Thr Lys Cys Gly Gly Asn
215   220   225   230

Gly Trp Cys Gly Ala Asp Arg Ile Asp Ala Trp Gly His Gly Thr Glu Val Ile
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 1   5   10   15

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              20   25   30

Lys Asp Pro Ser Ser Arg Ser Ala Ala Gly Thr Met Glu Phe Arg
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Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys
              50   55   60

Ala
61

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 1   5   10   15

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              20   25   30

Leu Phe Ile Asn Phe Cys Leu Ile Leu Ile Cys Leu Leu Leu Ile
            35   40   45

Cys Ile Ile Val Met Leu Leu His His His His His His Lys Gly
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Trp Lys Lys Ala
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Leu Phe Ile Asn Phe Cys Leu Ile Leu Ile Cys Leu Leu Leu Ile
            35   40   45

Cys Ile Ile Val Met Leu Leu His His His His His His Lys Gly
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Glu Phe Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys
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Trp Lys Lys Ala
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                20   25   30

Leu Phe Ile Asn Phe Cys Leu Ile Leu Ile Cys Leu Leu Leu Ile
                35   40   45

Cys Ile Ile Ala Met Leu Leu His His His His His His Lys Gly
                50   55   60

Glu Phe Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys
                65   70   75

Trp Lys Lys Ala
                79

(54) Title: A METHOD FOR INHIBITION OF PHOSPHOLAMBAN ACTIVITY FOR THE TREATMENT OF CARDIAC DISEASE AND HEART FAILURE

(57) Abstract

The present invention provides a method for the treatment of heart failure through the use of small peptide complexes and recombinant proteins which function to enhance contractility in failing hearts and reduce blood pressure in individuals with hypertension by inhibiting the interaction between phospholamban and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) within cardiomyocytes. In addition, a means is provided for the transport of such therapeutic agents into the cytoplasm and nucleus of cardiomyocytes.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   A61K38/17   A61K47/48   C07K17/00   A61P9/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   A61K   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOSS K L ET AL: "PHOSPHOLAMBAN: A PROMINENT REGULATOR OF MYOCARDIAL CONTRACTILITY" CIRCULATION RESEARCH, vol. 79, no. 6, 1 December 1996 (1996-12-01), pages 1059-1063, XP002053754 ISSN: 0009-7330 the whole document	1-5, 12-17
X	DILLMANN W H: "Influences of increased expression of the Ca2+ ATPase of the sarcoplasmic reticulum by a transgenic approach on cardiac contractility." ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, (1998 SEP 16) 853 43-8. , XP000914521 page 46, line 47 -page 47, line 16	1-5, 12-17

-/--

| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of ma q of the international search report
5 June 2000	20/06/2000

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INTERNATIONAL SEARCH REPORT

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Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	HE HUAPING ET AL: "Effects of mutant and antisense RNA of phospholamban on SR Ca2+-ATPase activity and cardiac myocyte contractility." CIRCULATION , vol. 100, no. 9, 31 August 1999 (1999-08-31), pages 974-980, XP000907563 ISSN: 0009-7322 the whole document ___	1-17
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CORRECTED VERSION

(51) International Patent Classification[7]: A61K 38/17, 47/48, C07K 17/00, A61P 9/04

(21) International Application Number: PCT/US99/25692

(22) International Filing Date:
2 November 1999 (02.11.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/106,718   2 November 1998 (02.11.1998)   US
60/145,883   27 July 1999 (27.07.1999)   US

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(81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

(88) Date of publication of the international search report:
5 October 2000

(48) Date of publication of this corrected version:
6 December 2001

(15) Information about Correction:
see PCT Gazette No. 49/2001 of 6 December 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A METHOD FOR INHIBITION OF PHOSPHOLAMBAN ACTIVITY FOR THE TREATMENT OF CARDIAC DISEASE AND HEART FAILURE

(57) Abstract: The present invention provides a method for the treatment of heart failure through the use of small peptide complexes and recombinant proteins which function to enhance contractility in failing hearts and reduce blood pressure in individuals with hypertension by inhibiting the interaction between phospholamban and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) within cardiomyocytes. In addition, a means is provided for the transport of such therapeutic agents into the cytoplasm and nucleus of cardiomyocytes.

A METHOD FOR INHIBITION OF PHOSPHOLAMBAN ACTIVITY FOR THE TREATMENT OF CARDIAC DISEASE AND HEART FAILURE

This application claims the benefit of priority of United States

5   Provisional Application Serial No. 60/106,718, filed November 2, 1998 and United States Provisional Application Serial No. 60/145,883, filed July 27, 1999, both of which are incorporated herein by reference in their entirety.

10   **BACKGROUND OF THE INVENTION**
                              FIELD OF THE INVENTION

This invention relates generally to a method for the treatment of heart failure, and more specifically to the inhibition of phospholamban (PLB) activity for the treatment of myocardial dysfunction.

15

                              BACKGROUND INFORMATION

Heart failure is the leading cause of combined morbidity and mortality in the United States and other developed countries. Congestive heart failure is characterized by a reduced contraction and delayed

20   relaxation of the heart however, fundamental molecular mechanisms which drive the patho-physiological pathways for congestive heart are largely unknown. Current therapy for the heart disease is primarily palliative and is not targeted to the underlying biological pathways which are thought to lead to the initiation and progression of cardiac muscle

25   dysfunction.

Heart muscle failure is a complex, integrative, multi-factorial disease in which the genetic pathways that confer susceptibility are interwoven with the environmental stimulus of biomechanical stress that accompanies heart injury, pressure and volume overload, and genetic defects in

30   components of the cytoskeleton. In response to this biomechanical

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stress, a series of parallel and converging signaling pathways are activated that lead to the adaptive response of compensatory hypertrophy. Subsequently, there can be a transition to chamber dilation and pump failure that is associated with a loss of viable myocytes, a decrease in

5   contractile elements, myofilament disarray and interstitial fibrosis.

Recently, the activation of signal transduction pathways which trigger the onset of programmed cell death have been implicated in promoting the pathological transition to heart failure, as well as a gp130 dependent myocyte survival pathway that can block the actions of the

10   pro-apoptotic pathways and prevent the early onset of heart failure and cardiomyopathy. In addition to these extrinsic stress-related pathways for myocyte adaptation, there also must be intrinsic-signaling pathways that   · lead to the impairment of cardiac excitation-contraction (EC) coupling and associated severe defects in cardiac contractile performance that are the

15   clinical hallmarks of the progression of the heart failure phenotype.

The sarcoplasmic reticulum (SR) plays an integral role in the coordination of the movement of cytostolic Ca^{2+} throughout the cardiac tissue. In separate studies by Mercadier, et al. (J. Clin. Invest.,1990; 85:305-309), Arai, et al. (Circ. Res., 1993; 72:463-469), de la Bastie, et

20   al. (Circ. Res., 1990; 66:554-564), and Feldman, et al. (Circ. Res., 1993; 73:184-192), research on human failing hearts and animal models of heart failure have suggested that the reduced uptake the cytostolic Ca^{2+} by the SR is responsible for prolonged diastolic relaxation. Ca^{2+} stored in the SR is released into the cytosol to activate the contraction of cardiac muscle

25   and subsequently re-accumulated to achieve relaxation. Activity of the cardiac SR Ca^{2+} ATPase (SERCA2a) is the rate determining factor of Ca^{2+} re-uptake into the SR, and SERCA2a activity is itself regulated by phospholamban, a 52-amino acid muscle-specific SR phosphoprotein.

Phospholamban (PLB) was first identified as a major

30   phosphorylation target in the SR membrane in research by Tada, et al. (J.

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Bio.l Chem., 1974; 249:6174-6180) and appeared to be a potent inhibitor of SERCA2a activity in its unphosphorylated form. The inhibitory effect of PLB on SERCA2a is reduced by an increase in intracellular calcium or by the phosphorylation of PLB in response to β-adrenergic stimulation. PLB

5   exists primarily in a pentameric form, that when subjected to high temperature, dissociates into five equivalent monomers.

The amino acids of monomeric PLB are grouped into three physical and functional domains. Domains Ia and II are rich in α-helices and are connected by the less structured domain Ib. Domain Ia is composed of

10   amino acids 1-20, the majority of which are in an α-helical confirmation, having a net positive charge. Domain Ib consists of amino acid residues 21-30 and constitutes the cytoplasmic sector of the monomer. Domain II amino acids 31-52, represents the transmembrane sector and is made up solely of uncharged residues that are responsible for stabilizing the

15   pentamer formation.

PLB is a mediator in the regulation of myocardial function by catecholamines through the cAMP cascade. Ser (16) and Thr (17), in domain Ia are the confirmed binding sites for cAMP-dependent protein kinase (PKA) and Ca/calmodulin-dependent protein kinase, respectively,

20   which function to catalyze phosphoester phosphorylation of PLB which in turn relieves its inhibition on SERCA2a activity. Because Ser (16) and Thr (17) can be phosphorylated by the kinases, the net charge of the amino acids can be shifted from positive to neutral and even to negative. Together with the charged residues of SERCA2a, the shifting of charges

25   in domain Ia of PLB can result in profound alterations in the protein-protein interaction of the PLB-SERCA2a system. Domain II also contains some key amino acids for functional expression of PLB, in that amino acids of one face of the domain II helix are associated with the transmembrane domain of SERCA2a.

30   There may be two ways in which PLB regulates Ca^{2+}-ATPase

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activity: 1) a quick-acting, short-term mechanism involving PLB

phosphorylation and depression of calcium pumping activity, and 2) a

slower acting but longer term process involving a change in the molecular

ratio of PLB to the Ca^{2+}-ATPase brought about by control of gene

5   expression. Under physiological conditions, phosphorylation at Ser (16)

by PKA is the predominant event that leads to proportional increases in

the rate of Ca^{2+} uptake to the SR and accelerates ventricular relaxation.

An increase in the relative ratio of PLB to SERCA2a is an important

determinant of SR dysfunction in both experimental and human heart

10   failure. Moreover, attenuated PLB phosphorylation by PKA may be

responsible for impaired diastolic function and prolonged Ca^{2+} transients

in failing hearts by which the β-adrenergic receptor-cAMP system is

severely down-regulated by enhanced sympathetic tone.

A detailed mutagenesis study by Toyofuku, *et al.* (*J. Biol. Chem.*,

15   1994; 269:3088-3094) revealed that several amino acids in the

cytoplasmic domain of PLB are important for its inhibitory function. The

study showed that when the certain amino acids were mutated into amino

acids of different charge, the PLB mutants lost their inhibitory effect on

the co-transfected SERCA2 in HEK293 cells. However, it is still unclear

20   whether PLB bearing these mutants can exert dominant negative effects

on endogenous wild-type PLB and consequently stimulate endogenous

SERCA2a in cardiac myocytes. Additionally, it is unclear how the

mechanisms of transfer of these PLB point mutations into cardiomyocytes

breach the cytoplasmic membrane barrier in order to effect endogenous

25   SERCA2a activity.

Genetically based mouse models of dilated cardiomyopathy by

Arber, *et al.* (*Cell*, 88:393-403; 1997) provide evidence that chamber

dilation and the progression to heart failure is dependent on a specific

Ca^{2+} cycling defect in the cardiac sarcoplasmic reticulum. In the mouse

30   models, ablation of phospholamban (PLB) rescued the spectrum of

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structural, functional, and molecular phenotypes that resemble heart failure. Furthermore, release of the phospholamban-SERCA2a interaction through the forced *in vivo* overexpression of a PLB point mutation dominantly activated the contractility of ventricular muscle cells. Thus,

5   there is the possibility that interfering with the PLB-SERCA2a interaction may provide a novel therapeutic approach for preventing heart failure.

There is the understanding that interfering with the PLB-SERCA2a interaction may be a potential therapeutic target for the treatment of heart failure, however, the internalization of exogenous molecules to enhance

10   cardiac contractility by live myocytes remains an unsolved issue. A means must be available to deliver any therapeutic agent directly to the cytoplasm and nucleus of cardiac myocytes. Penetratins, a class of peptides with translocating properties, have the ability to carry hydrophillic compounds across the plasma membrane. Research by Schwarze, *et al.*

15   (*Science* 285:1569-1572; 1999) has demonstrated an approach to protein transduction using a penetratin-based fusion protein which contains an NH$_2$-terminal 11- amino acid protein transduction domain from the denatured HIV TAT protein (Genebank Accession No. AF033819) . Using this non-cell-type specific transfer system allows direct targeting of

20   oligopeptides and oligonucleotides to the cytoplasm and nucleus. One of the most well characterized translocation peptides is one that corresponds to residues 43 to 58 of antennapedia, a Drosophila transcription factor. It is believed that the translocation peptide interacts with charged phospholipids on the outer side of the cell membrane. Destabilization of

25   the bilayer results in the formation of inverted micelles containing the peptide that travels across the cell membrane and eventually open on the cytoplasmic side. While the use of transport peptides to move cargo molecules into cells is not novel, it has not been demonstrated that transport peptides work well in cardiomyocytes.

30   Thus the need remains for methods for the inhibition of PLB through

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the use of mutants or small molecule inhibitors of PLB in order to manipulate the PLB/SERCA2a interaction in cardiac myocytes, as well as a transport means for these mutants or small molecule inhibitors of PLB to cross sarcoplasmic reticulum membrane barriers into the cytoplasm of

5   cardiac myocytes for the treatment of cardiac disease and heart failure. The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

10   It is an advantage of the present invention to provide methods for treatment of heart failure by inhibiting the effect of phospholamban on Ca^{2+} uptake in cardiac tissue.

It is another advantage of the present invention to provide both small peptide complexes and recombinant proteins which function to

15   enhance contractility in failing hearts and reduce blood pressure in individuals with hypertension by inhibiting the interaction between phospholamban and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) within cardiomyocytes.

It is yet another advantage of the present invention to provide for a

20   family of compounds that consist of a transport peptide covalently attached to wild-type, mutant, or truncated PLB.

In a first exemplary embodiment of the present invention, recombinant adenoviruses are engineered which force the expression of wild-type or mutant forms of PLB which have the ability to selectively

25   interrupt the normal inhibitory interaction between PLB and SERCA2a and in turn dominantly activate cardiac contractility.

In a second exemplary embodiment of the present invention, contractilin, a recombinant adenoviral mutant of PLB (K3E/R14E), binds to and imitates phosphorylation of phospholamban. This leads to an

30   activation of the calcium pump of the sarcoplasmic reticulum thus

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increasing cardiac contractility.

In a third exemplary embodiment of the present invention, a compound consisting of a fusion of 1) a 16-residue transport peptide and 2) a truncated phospholamban protein or similar peptide are transported across the cell membranes in a receptor independent manner. Once inside the cytoplasm of the cardiomyocyte, the truncated phospholamban or similar peptide act as a competitive inhibitor of endogenous phospholamban interactions with SERCA2a.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is an illustration diagraming a working model for the role of the PLB-SERCA2a interaction in the progression of heart failure.

Figure 2 shows the hemodynamic analysis of rescue of in vivo cardiac dysfunction in DKO mice (a-d) and hemodynamic assessment of β-adrenergic response to progressive infusion of dobutamine (e-h), where Figure 2a shows the plot for maximal first derivative of LV pressure, LV dP/dtmax. Figure 2b shows the plot for minimal first derivative of LV pressure, LV dP/dtmin. Figure 2c shows the plot for Lv end diastolic pressure. Figure 2d shows the plot for Tau. Figure 2e shows a graph of maximal first derivative of LV pressure, LV dP/dtmax. Figure 2f shows a graph of minimal first derivative of LV pressure, LV dP/dtmin. Figure 2g shows a graph of Lv end diastolic pressure. Figure 2h shows a graph of heart rate.

Figure 3 shows plotted data from the analysis of rescue of physiological calcium signaling defects in DKO myocytes, where Figure 3a is a series of graphs of representative intracellular Ca^{2+} transient in WT, MLPKO and DKO myocytes. Figure 3b is a bar graph of the amplitude of Ca^{2+} transients. Figure 3c is a bar graph of intracellular diastolic Ca^{2+} concentration. Figure 3d is a bar graph of SR Ca^{2+} content. Figure 3e is the immunoblot of MLP deficiency.

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Figure 4a shows a dot blot analysis of rescue of embryonic gene markers of the heart failure phenotype in DKO mice. Figure 4b is a bar graph comparing the relative induction of mRNA for wild-type, MLPKO, and DKO myocytes.

5   Figure 5 illustrates the inhibition of the interaction between PLB and SERCA2a, where Figure 5a is a series of graphs which plot the length change in myocytes. Figure 5b is a summary of the data of cell length changes.

Figures 6a is a Western blot analysis of myocytes containing the

10   adenovirus transgenes expressing sense PLB (sPLB), antisense PLB (asPLB), E2A, R14E, S16N, and K3E/R14E against monoclonal PLB. Figure 6b is a Western blot showing the results of cell infectivity by PLB, sPLB and K3E/R14E. Figure 6c illustrates the Western blot analysis of PLB infectivity of Sol8 cells.

15   Figure 7 shows a plot of SR Ca 2+ uptake in homogenates of neonatal rat cardiomyocytes infected with adenovirus expressing the indicated genes.

Figure 8 shows a plot of the data derived from indo 1 fluorescence-facilitated Ca 2+ transients of myocytes.

20

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

To directly assess the role Ca^{2+} cycling defects play in the transition to heart failure, cardiomyopathy in muscle-specific LIM protein (MLP)-deficient mice can be reversed by removing the gene that codes for

25   PLB. Mice which harbor an ablation of MLP show many of the phenotypic features of human dilated cardiomyopathy at the molecular, cellular, and physiological levels. A uniform feature of end-stage dilated cardio-myopathy is a marked increase is cardiac wall stress that is accompanied by thinning of the chamber wall and an accompanying decrease in cardiac

30   contractility and relaxation. Because calcium cycling is critical for both

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cardiac relaxation and contractility, defects in the pathway that control calcium uptake and release from the sarcoplasmic reticulum are prime candidates for driving the progression of heart failure.

5   The creation of mice which harbor a deficiency in PLB, in addition to MLP, exhibit rescue from all the phenotypic characteristics of human heart failure normally found in MLP single knock-out (MLPKO) mice. To determine whether the functional benefits associated with PLB ablation in MLPKO mice specifically reflect the loss of the direct interaction between PLB and SERCA2a, the applicant of the present invention has engineered
10   point mutations in the PLB gene which interrupt the functional inhibitory interaction between PLB and SERCA2a. Through the creation of recombinant adenoviruses encoding point mutations in PLB, it is demonstrated that progressive defects in excitation-contraction coupling in heart failure are related to the enhanced inhibition of SERCA2a by PLB
15   and that the introduction of phospholamban deficiency into the setting of a transgenic model of cardiac hypertrophy results in rescued cardiac function. These results are independently supported by the fact that MLP-deficient mice harboring a transgene which directs cardiac specific overexpression of SERCA2a also exhibit rescue of the cardiomyopathy
20   phenotype. Taken together, these results provide clear evidence that sarcoplasmic reticulum calcium cycling is critical to the progression of heart failure and points to the critical regulatory role of PLB inhibition of SERCA2a activity in the progression of heart failure. This, in turn, pinpoints the possibility of PLB as a key therapeutic target.

25   Further study of the MLP-PLB knock-out (DKO) mice indicated that the induction of PLB deficiency in the setting of cardiomyopathic mutation can result in maximal stimulation of cardiac contractile performance. The contractility of the DKO hearts at baseline levels was comparable to the contractility of wild-type hearts following maximal β-adrenergic
30   stimulation. This result suggests that following the removal of the tonic

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inhibition of SR calcium pump function by PLB, there is essentially a "supra-rescue" in terms of cardiac contractile function of the cardiomyopathic heart. Since PLB is a direct substrate for phosphorylation by both cyclic AMP-dependent protein kinase A and calcium/calmodulin

5 dependent kinase, the regulation of cardiac contractility by cAMP-dependent stimuli may occur via the phosphorylation of PLB, which in turn prevents the inhibitory interaction with SERCA2a.

According to the theory behind the phosphorylation of PLB, the "supra-rescue" of the cardiomyopathic MLP-deficient mice to super-normal

10 levels in the setting of PLB-deficiency might simply reflect the removal of the rate limiting step in the tonic inhibition of cardiac contractility. Consistent with this rationale, studies by Rockman, *et al. (Proc. Natl. Acad. Sci. USA* 95:7000-7005; 1998) have documented that relief of β-adrenergic desensitization in the MLP-deficient mice can also lead to

15 significant rescue of the dilated cardiomyopathic phenotype. Since PLB is an SR protein that interacts with at least three regulatory components (cAMP-dependent protein kinase, calcium/calmodulin-dependent kinase, and protein phosphatase), it should be determined whether the dominant effect of PLB ablation on improving cardiac contractile performance

20 reflects the chronic interaction of PLB with the SERCA2a or whether this rescue effect is related to the interaction of PLB with other known or novel cardiac proteins.

Utilizing recombinant adenoviruses which force the expression of wild-type and mutant forms of PLB, the present invention provides for

25 point mutations in PLB that can selectively interrupt the normal inhibitory interaction between PLB and SERCA2a and can dominantly activate cardiac contractility in cardiac ventricular muscle cells in the absence of catecholamine. Figure 1 outlines the mechanism responsible for the rescue effect observed in the DKO mice. Both PLB and MLP are muscle-

30 specific proteins, and as such, there must be a muscle cell autonomous

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pathway that is required for the progression and the rescue of the
phenotype, as opposed to extrinsic stress signals that promote or
suppress myocyte survival pathways. Since PLB and MLP do not directly
interact at the protein level, eliminating direct interaction between PLB and
5   MLP as the basis for rescue, there must be a physiological as opposed to
biochemical regulatory pathway that links the PLB regulatory pathways
with the onset of dilated cardiomyopathy. As shown in Figure 1, in the
normal heart, SR-calcium stores are maintained through the activity of the
SERCA2a which leads to an uptake of calcium into the SR and
10   consequent maintenance of normal cardiac relaxation and reduction in wall
stress. Subsequently, SR release of calcium, via the calcium release
channel, results in normal quantal calcium release and the consequent
activation of the cardiac myofilaments leading to myocardial contraction.
Accordingly, enhanced Ca^{2+} content in the SR leads to an enhanced Ca^{2+}
15   release with a corresponding increase in cardiac contractility.

The activity of SERCA2a is regulated by the inhibitory effects of the
direct interaction of PLB with SERCA2a, which can be relieved by the
cAMP-dependent phosphorylation of PLB following the delivery of β-
adrenergic stimuli. In the setting of heart failure, there is a relative
20   decrease in SERCA2a function due to an inhibitory effect of PLB that
arises due to blunted β-adrenergic responsiveness. As a result, there is
less phosphorylation of PLB and a constitutive inhibition of SERCA2a, via
chronic interaction with PLB, leading to a relative decrease is SR calcium
content versus normal levels. This decrease in calcium stores is
25   translated into a decrease in the quantal release of calcium through the
calcium release channel and a consequent decrease in the single cell
calcium transients and *in vivo* cardiac contractility. In the DKO mice, the
inhibitory effect of PLB is removed, as shown in Figure 1, thereby
relieving the system from the downstream inhibitory effects of PLB on the
30   SR calcium pump, resulting in maintenance of SR calcium uptake and

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reduction of wall stress towards normal levels. At the same time, this increase in SR calcium content results in maintenance of normal calcium quantal release, thereby leading to maintenance of normal contractility and relaxation.

5

Muscle-specific LIM protein knock-out and Double knock-out mice

Tests of the present invention were conducted using a double knock-out (DKO) mouse model which harbors homozygous ablation of two independent muscle specific genes. For this strategy, PLB knock-out
10   (PLBKO) mice are mated into the background of MLP knock-out (MLPKO) mice which harbor molecular, structural and physiological features of the complex *in vivo* heart failure phenotype of dilated cardiomyopathy. The F3 generation of these mice are used for the actual experimentation to eliminate any potential background effects from either the PLBKO or
15   MLPKO line on the observed cardiac phenotype of the DKO line.

MLPKO mice display a marked increase in heart/body weight ratio (6.34 ± 0.22 mg/g, n = 8) versus age and gender matched wild-type mice (4.60 ± 0.21 mg/g, n = 7; $p < 0.001$). The heart/body weight ratio in DKO mice (5.13 ± 0.19 mg/g, n = 9) is significantly smaller than that of
20   MLPKO mice ($p < 0.01$) and is not statistically different from wild-type. To evaluate whether the decreased heart weight in DKO mice is associated with amelioration of the disrupted cytoskeletal phenotype observed in MLPKO mice, electron microscopic analysis is made of hearts from MLPKO and DKO littermates. Ablation of PLB in the background of
25   MLP $^{-/-}$ rescues the wide spectrum of ultrastructural defects originally observed in the MLP deficient hearts, including myofibrillar disarray and massive fibrosis. These data suggest that ablation of PLB prevents not only the increase in total heart mass, but also prevents the disorganization of cardiomyocyte cytoarchitecture and fibrosis in MLPKO cardiomyopathic
30   mice.

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To evaluate whether the marked decreases observed in *in vivo* global cardiac function is rescued in DKO mice, echocardiography is performed with age-matched littermates. As noted in Table 1, prevention of ventricular dilation is confirmed in DKO mice. MLPKO mice have

5   enlarged cardiac chambers, as revealed by increased left ventricular end-diastolic dimensions (LVEDD) and end-systolic dimensions (LVESD), whereas DKO mice have LVEDD in the normal range. The percent fractional shortening (%FS) and mean velocity of circumferential fiber shortening (mean Vcf), indicators of systolic cardiac function, are

10   improved in age-matched DKO littermates, also noted in Table 1. When compared to non-littermate wild-type mice as controls, most of the echocardiographic data in DKO mice are similar to those in wild-type mice, although %FS is slightly decreased in DKO mice. Furthermore, cardiac function of DKO mice remain in the normal range beyond 6 months of age

15   (n = 2). Despite the reduction of chamber dilation, there appears to be some hypertrophy in DKO hearts. The ratio of LVEDD to LV posterior wall thickness is markedly decreased in DKO mice, indicating that the wall stress of DKO mice is reduced in comparison to MLPKO or wild-type mice. These results indicate that global cardiac function of DKO mice is

20   preserved in the range comparable to the parameters of control hearts. It should be noted that mice which are heterozygous for PLB deficiency display an intermediate level of functional rescue versus the MLPKO and DKO mice, suggesting that partial ablation of PLB can lead to significant functional improvement of the heart failure phenotype in MLPKO mice.

25

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Table 1

Echocardiographic assessment

	MLPKO n = 12	MLPKO/PLBhet n = 12	DKO n = 10	wild-type n = 10
LVEDD (mm)	4.87 ± 0.14	4.10 ± 0.15***	3.75 ± 0.12***	3.89 ± 0.11***
LVESD (mm)	3.94 ± 0.18	3.06 ± 0.22***†	2.60 ± 0.10***	2.44 ± 0.07***
FS(%)	19.4 ± 1.7	26.2 ± 3.2*‡	30.6 ± 1.8***†	37.2 ± 1.4***
SEPth (mm)	0.68 ± 0.05	0.86 ± 0.05*†	0.83 ± 0.05*‡	0.64 ± 0.01
Pwth (mm)	0.67 ± 0.05	0.86 ± 0.05*‡	0.81 ± 0.04*†	0.64 ± 0.06
LVEDD/ Pwth(mm/mm)	7.47 ± 0.30	5.00 ± 0.47***	4.74 ± 0.30***	6.14 ± 0.18*
mean Vcf (circ/s)	3.38 ± 0.33	4.81 ± 0.73#	6.10 ± 0.41***†	4.41 ± 0.61
mean Vcfc (circ/s)	1.41 ± 0.17	1.96 ± 0.27*	2.37 ± 0.13***	2.23 ± 0.10**
Heart Rate (beats/min)	351 ± 15	350 ± 15§	396 ± 13§	239 ± 14***
Age (days)	65.3 ± 2.5	65.5 ± 4.5‡	67.4 ± 1.9‡	80.5 ± 1.2**
Body Weight (g)	26.7 ± 1.3	26.8 ± 0.9§	25.0 ± 1.3§	36.9 ± 2.2***

MLPKO vs. MLPKO/PLBhet or DKO or wild-type; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$

wild-type vs. MLPKO/PLBhet or DKO; †: $p < 0.05$, ‡: $p < 0.01$, §: $p < 0.001$

MLPKO/ PLBhet vs. DKO; #: $p < 0.05$

MLPKO mice have demonstrated a marked blunting of β-adrenergic
responsiveness and decreased adenyl cyclase activity. Ablation of PLB by
homologous recombination in mice can augment cardiac contractile
performance to a level comparable to that with maximal β-adrenergic
stimulation of the normal heart. To confirm that the ablation of PLB can
reverse the hemodynamic defects and marked β-adrenergic receptor
desensitization associated with dilated cardiomyopathy, anesthetized mice
are cardiac catheterized and assessed.

Several independent hemodynamic parameters document the rescue
of the severe cardiac dysfunction with circulatory congestion to normal
levels in the DKO mice. LV contractility (assessed by LV dP/dtmax) and

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relaxation (assessed by LV dP/dtmin) at baseline appears to be higher than that of wild-type mice and is comparable to PLBKO mice, as is shown in Figures 2a and 2b. Ablation of PLB reverses the markedly elevated LV end diastolic pressure observed in the MLPKO cardiomyopathic mice, as
5   seen in Figure 2c.

Analysis of Figure 2d illustrates that Tau, an indicator of LV relaxation and diastolic function, are also normalized in the DKO mice, which is consistent with an improvement in wall stress. These data suggest that ablation of PLB can rescue both the systolic and diastolic
10   dysfunction in the cardiomyopathic MLPKO mice. The blunted responses of LV dP/dtmax and LV dP/dtmin to β-adrenergic stimulation is observed in MLPKO mice, as shown in Figures 2e and 2f, indicating the presence of severe β-adrenergic desensitization in MLPKO mice. There is no stimulation of cardiac contractility (LV dP/dtmax) and relaxation (LV
15   dP/dtmin) by dobutamine in DKO mice, as is again shown in Figures 2e and 2f. These parameters are already stimulated to their maximal levels under basal conditions in the DKO hearts.

After maximum stimulation of wild-type hearts by dobutamine, LV dP/dtmax and LV dP/dtmin are indistinguishable from these parameters in
20   the DKO mice in the absence of any catecholamine stimulation. This evidence confirms that the interaction of PLB and SERCA2a suppresses cardiac contractility in both normal and myopathic hearts, and that inhibiting this interaction may exert a dominant effect on maximizing cardiac performance in the absence of any catecholamine stimulation.
25   Figure 2h shows that chronotropic responsiveness to dobutamine is preserved in both DKO mice and MLPKO mice, thereby documenting the specificity of the β-adrenergic response to ventricular myocytes versus pacemaker cell types.

To determine the mechanisms responsible for the rescue of *in vivo*
30   cardiac function in DKO mice, several independent parameters of Ca^{2+}

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signaling are assessed. It is apparent that altered Ca^{2+} homeostasis in DKO mice leads to the hemodynamic changes, intracellular Ca^{2+} transients and the expression of proteins related to Ca^{2+} cycling in the SR. MLPKO myocytes exhibit an attenuated amplitude of Ca^{2+} transients with normal

5   levels of diastolic Ca^{2+} concentration, as is show in Figures 3a-c. The rate of decay is slightly accelerated in MLPKO mice which suggests that a compensatory mechanism is operative during the end of Ca^{2+} uptake in MLPKO mice. Ablation of PLB is associated with a shortened duration of the Ca^{2+} transient, faster decay and preserved amplitude. Figure 3d

10   shows that SR Ca^{2+} content is significantly decreased in MLPKO mice and increased in DKO mice as compared to wild-type mice. Quantitative immunoblotting, shown in Figure 3e, reveals that MLP deficiency is not associated with any significant alterations in protein levels of SERCA2a, PLB and calsequestrin, suggesting that the defects of Ca^{2+} cycling in

15   MLPKO mice is based upon functional impairment of EC coupling, as opposed to simply reflecting decreases in the protein levels of either SERCA2a or phospholamban.

One of the characteristic features of heart failure is the reactivation of an embryonic gene program which may contribute to a compensatory

20   response to an increased hemodynamic load. To confirm that hemodynamic improvement in DKO mice is accompanied by amelioration of changes at the transcriptional level, the expression of ANF, α-skeletal actin, and β-MHC mRNAs, well established embryonic markers for heart failure, is examined. As shown in Figures 4a and 4b, ventricles of MLPKO

25   mice display a 26-fold increase in ANF, a 13-fold increase in α-skeletal actin and an 8-fold enhancement of β-MHC mRNAs. DKO mice exhibits only a 1.9-fold increase in ANF and no significant increase in α-skeletal actin or β-MHC mRNAs. Thus, ablation of PLB largely suppresses induction of the embryonic gene program in MLPKO mice.

30   The aforementioned studies indicate that the ablation of PLB can

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rescue independent parameters of heart failure and associated defects in cardiac contractility. To define the mechanism of the rescue effect, it is necessary to assess whether the chronic interaction of PLB and SERCA2a is in fact limiting for cardiac contractility in both normal and myopathic

5   hearts. The "supra-rescue" of basal cardiac function in the DKO mice to levels comparable to those in wild-type mice following maximal catecholamine stimulation suggests that inhibition of this interaction may exert a dominant effect to maximize cardiac performance in the absence of any catecholamine stimulation.

10

Recombinant Adenoviral transgene mutants of PLB

Using the knowledge that certain amino acid residues of PLB are required to maintain its inhibitory effects on SERCA2a, several single point mutations of PLB, V49A (Seq. ID. No. 2), E2A (Seq. ID. No. 3), R14E

15   (Seq. ID. No. 4), S16N (Seq. ID. No. 5),a double point mutation of PLB, K3E/R14E (Seq. ID. No. 6) and a sense and antisense PLB (Seq. ID. No. 1) transgene can be engineered in order to disrupt the inhibitory effects of PLB on SERCA2a. Using recombinant adenoviruses for *in vivo* murine cardiac gene transfer, myocytes which overexpresses V49A, one of the

20   single point mutations in PLB, exhibit an increase in contractility, while myocytes which overexpress the wild-type PLB exhibit a decrease in contractility when compared to non-infected myocytes, as is documented in Figure 5. It can be concluded that the feasibility and utility of interfering with the interaction between the SERCA2a and PLB is clearly

25   documented. The PLB-SERCA2a interaction appears to be the rate limiting step for establishing the set point of basal cardiac contractility and relaxation in vivo, and the disruption of this interaction can thereby short circuit the β-adrenergic pathway.

Additional Western blot analysis of myocytes containing the

30   adenovirus transgenes expressing sense PLB (sPLB), antisense PLB

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(asPLB), E2A, R14E, S16N, and K3E/R14E against monoclonal PLB antibody (Affinity BioReagents) is shown in Figure 6a. Quantification of PLB protein content, normalized to α-actin for loading variance and compared with an adenovirus/ SR control lacking the transgene, shows

5   that sPLB, E2A, and R14E mutants increase PLB protein level by 150% (PLB_5 + PLB_1), 72 %, and 57%, respectively. In contrast, asPLB and S16N results in 54% and 33% decrease in PLB protein content within the myocytes. The introduction of K3E/R14E transgene infection of myocytes leads to a formation of a distinct pattern of pentamer PLB. Multiple PLB

10   bands appear in addition to PLB_5 (the pentamer). This is accompanied by a reduced abundance of PLB_5 in comparison with the control.

The nature of the Western blot banding pattern is further evaluated by substituting PLB-deficient Sol8 cells in place of cardiac myocytes. Sol8 cells are infected with the recombinant adenovirus expressing either

15   the transgene sPLB or K3E/R14E alone or in combination. As seen in Figure 6b, the Western blot shows that the monoclonal PLB antibody detects PLB in cells infected by sPLB but fails to detect K3E/R14E. Infection of Sol8 cells with a combination of the adenoviral transgenes results in formation of multiple bands of PLB. Moreover, the PLB

20   pentamer decreases in abundance simultaneously with the appearance of the upper bands. It is well established that PLB interacts with and inhibits SERCA2a predominantly as a monomer that exists in equilibrium with the noninhibitory pentamer. Based on this knowledge, the heteropentamer of K3E/R14E and wild-type PLB might be more stable that the

25   homopentamer of wild-type PLB. Therefore, the dissociation of the heteropentamer into monomers, which results in inhibition of SERCA2a, is disfavored. K3E/R14E interacts with endogenous PLB and forms such a complex, accompanied by a decrease in homopentamer formation. In as much, the monomer K3E/R14E may act as a noninhibitory competitor for

30   endogenous wild-type PLB by blocking SERCA2a-PLB interaction sites.

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The effects of mutant and antisense PLB on SERCA2a is further evaluated by determination of the SR calcium uptake activity. The initial rate of Ca^{2+} uptake by the SR measured at varying Ca^{2+} concentrations reflects the activity of SERCA2a. As shown in Figure 7, neonatal rat

5   myocytes infected with the recombinant adenovirus transgenes K3E/R14E and asPLB show a decrease in the concentration of Ca^{2+} needed by SERCA2a for the same activity compared with the non-transgene control, indicating a stimulation of SERCA2a activity. The EC_{50} s of Ca^{2+} concentration at which the uptake activity is half-maximal are, (in μmol/L),

10   0.20 ± 0.02 for the non-transgene control (SR-), 0.11 ± 0.01 for K3E/R14E, and 0.13 ± 0.01 for asPLB. The effects of K2E/R14E and asPLB on SERCA2a are also examined in adult rat myocytes. The adenoviral transgene K3E/R14E lowers the EC_{50} significantly (by 36%), whereas the change as a result of asPLB infection is not within statistical

15   significance. This apparent discrepancy in the effects between neonatal and adult cardiac myocytes is possibly related to the different abundances of PLB in myocytes at different developmental stages. PLB is nearly twice as abundant in adult as in neonatal myocardium.

To further examine the effects of K3E/R14E and asPLB on

20   SERCA2a, intracellular Ca^{2+} transients in neonatal myocytes are measured by use of the indo 1 fluorescence indicator. Indo 1 ratiometric data which is obtained from each of the experimental conditions is normalized to the respective maximum and minimum of each contractile Ca^{2+} transient and is then aligned and averaged. As shown in Figure 8, the decay curves of

25   K3E/R14E and asPLB are displaced to the left of the LacZ control. Furthermore, for most of the diastolic time points, K3E/R14E is significantly different from LacZ, whereas at several diastolic time points, asPLB is also significantly different from LacZ. The half-times for decay (RT_{50}) for LacZ, K3E/R14E, and asPLB are determined to be 0.28 seconds

30   (100%), 0.20 seconds (73%), and 0.22 seconds (79%), respectively.

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The values for K3E/R14E (73%) and asPLB (79%) are significantly different ($p < 0.05$) from the values obtained from the LacZ expressing virus.

In addition to generating adenoviral transgenes using various point mutations of PLB, or the sense or antisense sequences of PLB, antibodies raised against PLB peptide and then expressed as RNA can also be inserted into the adenoviral vector. To raise polyclonal PLB antibody ("contractilin", or chicken antibody peptides with hyperactive regions), a chicken is repeatedly immunized with PLB peptide which represents amino acids 3 to19 of the cytoplasmic domain. After three rounds of booster immunization, administered at 15, 42 and 54 days, total IgY is purified from the egg yolk, using a commercially available purification system (EGGstract IgY Purification System - Promega). Upon confirmation of a positive immune response, lymphocytes from the spleen and bone marrow are harvested. RNA, in the form of the hypervariable regions from both antibody light and heavy chain is obtained from these cells and amplified by RT-PCR, the method of which is well known.

The amplified and purified hypervariable region RNA is then fused to a single cDNA (Seq. ID. No. 9) and subsequently cloned in frame into a plasmid vector, coding for a phage surface protein. Using standard phage display technique, phages which express the immune library of the chicken are selected by their positive response to the PLB peptide. After a series of enrichment for phages which specifically bind PLB, 20 clones are selected for ELISA. The resulting 5 best binders are then analyzed using a radioactive Ca^{2+} transport assay. The two best activators of SR Ca^{2+} transport are further analyzed. Both clones are found to dramatically stimulate the rate of Ca^{2+} transport into the SR.

To demonstrate that the recombinant protein, which has been generated from contractilin (the PLB antibody), can also function inside a living cell, an adenoviral vector expressing contractilin is constructed.

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Western blot analysis of neonatal and adult rat cardiomyocytes, infected with the adenoviral transgene, shows that contractilin can be expressed in heart cells. Radioactive Ca^{2+} transport analysis indicate that, as with the mutant and antisense PLB, contractilin accelerates cytoplasmic Ca^{2+}

5   removal.

In a complementary approach, plasmid transfection rather than adenoviral transfection is used for gene delivery. It is found that K3E/R14E- and asPLB- transfected myocytes, as monitored by co-transfected green fluorescence protein, exhibits 43% ($p < 0.05$) and 9%

10   ($p < 0.1$) decreases in RT_{50}, respectively, relative to adenoviral vector transfected cells. Thus, introducing K3E/R14E and asPLB into the cardiac myocytes by either the adenovirus or co-transfection technique reduces the duration of the diastolic Ca^{2+} transients. These results would seem to mirror the findings of MLPKO versus DKO mice where variation in Ca^{2+}

15   transients confirm that ablation of PLB is associated with a shortened duration of Ca^{2+} transient, faster decay, and preserved amplitude. Taken together, these data confirm that K3E/R14E and asPLB stimulate the SERCA2a activity, which results if faster Ca^{2+} transients in myocytes.

To determine whether the enhanced SERCA2a activity and

20   accelerated Ca^{2+} transients, as a result of the PLB mutants, lead to change in contractile behavior, edge detection is used to analyze myocyte contractility. Adult rabbit myocytes are infected with the adenoviral transgenes of LacZ, K3E/R14E, or asPLB. After a three day incubation period, there is a significant difference in the number of spontaneously

25   contracting cells between the different groups (LacZ < < asPLB < K3E/R14E). Table 2 provides the effects of K3E/R14E and asPLB on myocyte contractility. As shown in the table, compared with the LacZ control, K3E/R14E increases fractional shortening by 74%, which is accompanied by a 25% decrease in RT_{50} and a 115% increase in $+dL/dt$.

30   When the myocyte contractility is examined after asPLB infection, it is

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found that the fractional shortening of the myocytes increases significantly, by 57%, whereas the changes in RT_{50} and $+dL/dt$ are not significant.

Table 2

	LacZ (n = 33)	K3E/R14E (n = 29)	asPLB (n = 30)
$+dL/dt$ ($\mu m/s$)	11.7 ± 1.9	$25.1 \pm 1.6*$	$18.4 \pm 2.0*$
RT_{50} (ms)	539.0 ± 27.0	$402.0 \pm 19.0*$	$483.0 \pm 29.0***$
Shortening (%)	6.2 ± 0.5	$10.8 \pm 0.5*$	$8.0 \pm 0.6***$

*: $p < 0.05$
**: $p < 0.1$

The resulting data show that the increase in SERCA2a activity translates into an accelerated relaxation of the myocytes. K3E/R14E-infected myocytes display an enhanced fractional shortening, which suggests an increase in SR loads of Ca^{2+} due to the enhancement of SERCA2a activity. Further, K3E/R14E infection increases the number of spontaneously contracting myocytes, a phenomenon most likely associated with the increased amount of oscillating Ca^{2+} due to the elevated SR loading of Ca^{2+}. Taken together, these data show that K3E/R14E affects endogenous wild-type PLB in a way that significantly reduces its inhibition of SERCA2a and thus has a dominant inhibitory effect over wild-type PLB.

Peptide-based therapeutic for inhibition of PLB activity

Still further, the present invention provides for a peptide based therapeutic for the inhibition of phospholamban activity and a mode of delivery for such a therapeutic, based on the finding that PLB function can be inhibited in a dominant negative manner by overwhelming endogenous PLB with mutant PLB molecules, and that this inhibition leads to improved function in failing hearts.

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For a therapeutic agent, such as an inhibitor of the PLB-SERCA2a interaction, to effect a target cell system, it must have a means for internalization through the cell membrane into the cytoplasm. The mode of transfer of the inhibitor can be by way of either a transport or

5   penetratin based PLB peptide or it can also include adenoviral or lipid vesicle based transfer. For this purpose, a compound consisting of a fusion of a transport peptide and a PLB protein molecule is constructed. The transport peptide comprises a 16-residue from the sequence for antennapedia, a Drosophila transcription factor protein. The second

10   peptide of the complex can be a truncated sequence of PLB protein. Further therapeutic benefits can be achieved using peptides that correspond to native PLB protein as well as a mutant or truncated form of PLB protein.

One beneficial function of the transport peptide-PLB complex is the

15   inhibition of the interaction between PLB and SERCA2a within cardiomyocytes, resulting in enhanced contractility in diseased hearts. The present invention may also inhibit the interaction of PLB with SERCA2a within the smooth muscle layer surrounding the arteries/ arterioles of the circulatory system which would result in vasodilation and

20   reduced blood pressure. Thus, there is a two-fold benefit in the treatment of heart disease, the first is enhanced cardiac contractility in failing hearts, the other is the reduction of blood pressure in individuals with hypertension. It is also predicted that there will be the inhibition of PLB interactions with SERCA proteins of other cell types, such as the

25   SERCA1-PLB interaction in nervous tissue.

The introduction of the molecule into the blood stream feeding the heart can is best achieved using a catheter located in the coronary artery. When the molecule is present in the extracellular environment surrounding a cardiomyocyte it rapidly enters the cardiomyocyte and inhibits the

30   association of PLB with SERCA2a. The translocation function is

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attributable to the transport peptide which exhibits the ability to rapidly translocate itself and the attached "cargo" peptides across the cell membranes in a receptor independent manner. Once inside the cytoplasm of the cardiomyocyte, the PLB fragment will act as a competitive inhibitor

5   of endogenous PLB interactions with SERCA2a.

In the absence of PLB inhibition by association, SERCA2a more efficiently pumps Ca 2 + into the SR, thereby increasing the cardiomyocytes ability to contract more strongly and rapidly. Stronger cardiomyocyte contractility translates to more powerful heart contractility.

10   In vivo, the present invention could act as a treatment for heart failure and is most easily administered and most effective in patients whose hearts require, or already have implanted, a left-ventricular assist device (LVAD).

While residues 43 to 58 of Antennapedia is a well characterized translocation peptide, and works well in the present invention, the present

15   invention is not restricted to this method of transport. Other potential methods of transfer include the use of an 8-branched polylysine backbone to link the transport and cargo peptide, but it is not limited to this multi-branched structure. A compound consisting of one target peptide attached to one PLB peptide, as one long peptide, has also been explored.

20   Still further, a number of DNA constructs for producing hexahistidine (H6) tagged penetratin and penetratin-PLB recombinant proteins in bacteria have been undertaken. The penetratin peptides were engineered to be on either the amino or carboxy terminal end of the protein.

It has been shown that the cytoplasmic fragment of PLB has as

25   strong a binding affinity for the cytoplasmic portion of SERCA2a as the whole PLB molecule. Therefore, once the transport-PLB molecule is inside the cytoplasm of the cardiomyocyte, the PLB fragment is predicted to act as a competitive inhibitor of endogenous PLB interaction with SERCA2a.

This form of treatment is suitable for the patient who is suffering

30   from severe decreased cardiac pump function, refractory to medical

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therapy, and requiring mechanical assistant devices while waiting for heart transplantation. In addition, the underlined molecular mechanisms for the dominant negative function of the PLB mutants can be used in the design and implementation of the high-throughput screening strategies for

5   inhibitory small molecules.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE 1

10   Creation of knock-out mouse lines for echocardiography

In order to analyze the structural and physiological features of the complex *in vivo* heart failure phenotype of dilated cardiomyopathy, several lines of knock-out mice were created. conducted using a double knock-out (DKO) mouse model which harbors homozygous ablation of two

15   independent muscle specific genes. For this strategy, PLB $^{-/-}$ (phospholamban deficient) homozygous mice were mated with MLP $^{-/-}$ (muscle- specific LIM protein) homozygous mice. The F1 pups generated from an MLP$^{-/-}$ x PLB$^{-/-}$ homozygote cross were then mated to create the MLP$^{+/-}$, PLB$^{+/-}$ double heterozygote genotype. F2 offspring were

20   generated from a MLP$^{+/-}$ / PLB$^{+/-}$ double heterozygote mating, thereby creating mice that were homozygous for the mutant MLP allele and heterozygous for the mutant PLB allele or that were MLP wild-type and heterozygous for the mutant PLB allele. The F3 offspring were generated from a MLP$^{-/-}$ / PLB$^{+/-}$ matings to generate MLP$^{-/-}$ / PLB$^{-/-}$ (DKO), MLP$^{-/-}$ /

25   PLB$^{+/+}$ (MLPKO) and MLP$^{+/+}$ / PLB$^{-/-}$ (MLPKO/ PLBhet) littermates or from a MLP$^{+/+}$ / PLB$^{+/-}$ matings to generate MLP$^{+/+}$ / PLB$^{-/-}$ (PLBKO), MLP$^{+/+}$ / PLB$^{+/+}$ (wild-type) and MLP$^{+/+}$ / PLB$^{+/-}$ (PLBhet) littermates. The genotype of the gene-targeted crosses were determined by PCR or genomic DNA isolated from tail biopsies.

30   To evaluate the hemodynamic properties of the various knock-out

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mouse lines, cardiac catherization and echocardiography was performed on subjects anesthetized with either Avertin (2.5%, 20 μl/kg body weight) or xylazine (0.005 mg/g) and ketamine (0.1 mg/g). Transthoracic M-mode echocardiographic tracings indicated that MLPKO mice had chamber

5   dilation with reduced wall motion, indicating depressed cardiac function and increased wall stress, whereas chamber size and cardiac function are normal in the DKO mice. Baseline parameters for wild-type (WT), n = 7, MLPKO, n = 8, DKO, n = 9, and PLBKO, n = 5 are shown in Figures 2a-d. Data were expressed as mean ± SEM. MLPKO versus other groups;

10   *p < 0.5, **p < 0.001, WT vs. DKO; #p < 0.01. In Figures 2e-h, hemodynamic assessment was made of β-adrenergic responsiveness to progressive infusion of dobutamine, where WT (□), n = 7, MLPKO (●), n = 8, and DKO (○), n-9, mice. MLPKO vs WT or DKO; #p < 0.05, +p < 0.01, *p < 0.001, WT vs DKO; ∋p < 0.01.

15

EXAMPLE 2

Calcium transient analysis

       To evaluate the effect of inhibition of PLB on SR calcium content and calcium transients, myocytes were isolated from the right ventricular

20   wall of the wild-type or knock-out mice. To monitor the changes in intracellular calcium, the isolated myocytes were incubated with a calcium sensitive dye, fluo-3-AM (1 μg/ml), for 30 minutes at room temperature. The myocytes were then transferred to a tissue chamber on the stage of an inverted microscope and continuously stimulated at a rate of 1 Hz to

25   maintain a consistent degree of SR calcium loading. To measure cellular fluorescence, the myocytes were illuminated with an excitation wavelength of 480 nm. Any changes in fluorescence were monitored at 510 nm using a microfluorometer (FM-1000; Solamere Technologies) and digitally recorded for later analysis using Cellsoft (D. Bergman; University

30   of Calgary) software. Fluorescence values were calibrated using the

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equation:

$$[Ca^{2+}]i = K_D(F-Fmin)/(Fmax-F) \qquad (1)$$

with an assumed K_D of 864 nM, where F are the experimentally derived fluorescence values. Fmax was determined by adding 10 μM ionomycin

5   to the superfusion solution and Fmin was determined by adding 4 mM $MnCl_2$ to the superfusion solution for each myocyte.

SR calcium content of the isolated myocytes was assessed using a standard caffeine pulse protocol. Following stable recordings of calcium transients, a 20 second pulse of 10 mM caffeine was applied to the

10   myocyte. This protocol resulted in a rapid caffeine-induced transient which slowly decayed back to baseline values. The SR calcium content was defined as the integrated area of this caffeine-induced calcium transient. Figure 3a illustrates the representative intracellular calcium transient in the WT, MLPKO and DKO myocytes. MLPKO myocytes

15   exhibited an attenuated amplitude of calcium transients with normal levels of diastolic calcium concentration. DKO myocytes displayed the calcium transient with a shortened duration, faster decay, and preserved amplitude. As shown in Figure 3b, the amplitude of calcium transient was significantly attenuated in MLPKO myocytes and was restored in DKO

20   myocytes. Figure 3c shows that intracellular diastolic calcium concentration was not different among the three different groups of myocytes. In Figure 3d, SR calcium content was significantly decreased in MLPKO mice and increased in DKO mice when compared to WT mice. In Figure 3e, representative quantitative immunoblotting revealed that MLP

25   deficiency was not associated with any significant alterations in the protein levels of SERCA2a, PLB, and calsequestrin.

EXAMPLE 3

Construction of mutant PLB adenovirus and gene transfer

30   I.M.A.G.E. consortium cDNA clones encoding human PLB were

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available through Genome System, Inc. The DNA fragment harboring the entire coding sequence of PLB was subcloned into pBluescriptII KS vector, as well known E. coli cloning vector (ATCC accession no. 87047). A sense mutation (Val49Ala) was introduced using a PCR based

5   mutagenesis system commercially available from Stratagene. Recombinant adenovirus expressing wild-type and mutant human PLB was generated by homologous recombination between plasmid pJM17 and a shuttle plasmid containing RSV promoter and SV40 poly A sequences. The concentrated virus preparation were tittered using a standard

10   protocol. The efficient *in vivo* cardiac gene transfer was performed by injecting the adenovirus vectors into 1 day old neonatal mouse heart. The myocytes were isolated 4 weeks after injection into the mouse hearts and cell shortening was measured. Myocytes harboring the mutant transgenes were identified by co-transfection of adenoviral vectors expressing GFP as

15   a marker.

EXAMPLE 4

Construction of a PLB inhibitor-transport peptide complex

A PLB inhibitor molecule was made by indirectly attaching a

20   transport peptide and a PLB protein to a polylysine backbone. Alternatively, the PLB molecule could also have been made as a single long peptide consisting of a transport sequence tandemly attached to the cargo peptide sequence. The transport peptide was composed of residues 43 to 58 of antennapedia (Seq. ID. No. 7), a Drosophila transcription

25   factor protein. The cargo peptide was derived using the first 16 residues of PLB (Seq. ID. No. 8). It is important to note that this cargo sequence could also have been derived from any segment of wild-type PLB or mutant PLB.

The PLB inhibitor molecule was constructed by linking 4 transport

30   peptides with 4 peptides matching the first 16 residues of PLB. The

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backbone linker was an 8-branch lysine, commonly used in multiple
antigenic peptide (MAP) synthesis. The first 4 branches of the MAP resin
were used to synthesize the antennapedia peptide. The next 4 branches
were then deprotected and used as the starting point for the synthesis of

5   the PLB cargo peptide. Thus, this particular PLB inhibitor that was used
for initial characterization had 4 branches of the antennapedia peptide and
4 branches of the PLB cargo peptide. Alternatively, the PLB inhibitor
could have been constructed as a single peptide with the cargo and
transport peptides attached to each other by a single peptide bond, or as

10   the cargo and transport peptides attached to each other by a disulfide
bond. The PLB inhibitor molecule was translocated efficiently into isolated
neonatal rat cardiomyocytes and showed a resulting enhanced
contractility of the cell, the results of which can be seen in Figures 5a and
b. Myocytes that overexpressed the V49A PLB point mutation showed

15   increased contractility, while myocytes which overexpressed the wild-type
PLB exhibited decreased contractility when compared to non-infected
myocytes.

EXAMPLE 5

20   Penetratin peptides TAT and ANT

          Cell level studies were done to evaluate the ability of two penetratin
-based peptides, two mutant PLB-penetratin peptides, and two multiple
antigen peptides (MAP) to strengthen the contraction cycle of isolated
mouse cardiomyocytes. The two penetratin-based peptides include PLB-

25   ANT (Seq. ID. No. 10) and TAT-PLB (Seq. ID. No. 11) each of which have
a 20 residue portion of the PLB sequence attached to either the 5' end of
ANT (Seq. ID. No. 14) or the 3' end of TAT (Seq. ID. No.15). The two
mutant PLB peptides, mutant PLB-ANT (Seq. ID. No. 12) and TAT-mutant
PLB (Seq. ID No. 13), display a S16E mutation of the 20 residue PLB

30   sequence. The multiple antigen peptides include MAP with 8 penetratin

-30-

(ANT) domains and MAP with 4 penetratin domains and 4 PLB domains .

Each of the penetratin-PLB peptides were evaluated to measure their ability to strengthen the contraction cycle of isolated mouse cardiomyocytes, the idea being that the penetratin-PLB peptide would act

5   as a dominant negative inhibitor of the PLB-SERCA2a interaction. The results of the TAT-PLB peptide on the isolated cardiomyocytes is shown in Table 3. For this data, tests were repeated on several sets of cardiomyocytes to determine relative change in length through the contraction cycle with the TAT-PLB peptide (samples 1-7) and without the

10   peptide (controls 1-8). Each of the samples had an added concentration of 10 μM of the TAT-PLB peptide while the controls had no added peptide.

Table 3

	Maximum Contract.	Minimum Contract.	% Contract.	Δ Length/ msec.	r2 (fit)	Contract. Slope	Relax. Slope
control 1	100.63	92.978	7.604	-34.503	0.9696	29.935	0.9955
control 2	91.146	83.301	8.607	-38.76	0.9943	28.736	0.9349
control 3	115.45	100.05	13.339	-82.875	0.9768	89.054	0.9894
control 4	105.00	102.04	2.819	-19.196	0.9842	10.497	0.9944
control 5	83.747	79.637	4.908	-21.695	0.9971	12.184	0.9742
control 6	145.96	136.85	6.241	-50.185	0.9721	27.566	0.9912
control 7	154.56	142.16	8.023	-76.607	0.9933	73.70	0.9928
control 8	115.59	102.68	11.169	-59.643	0.9765	63.304	0.9789
Mean	**114.010**	**104.962**	**7.839**	**-47.933**	**0.9830**	**41.872**	**0.9814**
sample 1	112.57	101.17	10.127	-65.554	0.9865	59.875	0.9925
sample2	109.68	102.00	7.002	-30.267	0.9609	37.157	0.9790
sample3	133.82	116.32	13.077	-79.242	0.9964	134.46	0.9878
sample 4	81.61	67.871	16.835	-58.093	0.9961	65.017	0.9697
sample 5	74.423	64.629	13.160	-54.353	0.9539	47.775	0.9933
sample 6	126.89	108.38	14.587	-98.054	0.9819	107.07	0.9939
sample 7	133.61	128.21	4.042	-36.071	0.9966	27.911	0.9959
Mean	**110.373**	**98.363**	**11.261**	**-60.233**	**0.9818**	**68.466**	**0.9874**

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Measurements were taken every 20 milliseconds, and the unit of length was arbitrary, but was generally on the order of one complete cell length. Percent contraction was calculated as (maximum length minus minimum length) divided by maximum length. A plot of time versus length

5   generated a U-shaped curve from which the most linear segments were selected, with the left side of the "U" representing contraction and the right side representing relaxation. The r2 column shows how well the data fit the curve, where1.0 represents a perfect fit.

While there appeared to be a trend towards a larger, faster

10   contraction in the myocyte, T-test analysis not identify any statistical difference due to the high variability of the data.

EXAMPLE 6

Hexahistidine tagged penetratin

15   A number of DNA constructs were generated for producing hexahistidine (H6) tagged penetratin and penetratin-PLB recombinant proteins in bacteria. Using a commercially available expression vector, pRSET (Invitrogen), recombinant protein H6-ANT (Seq. ID. No16) was generated. While this recombinant protein has no PLB attached, it was

20   engineered to have epitope tags which was used to detect the protein as it entered the heart. A variation of H6-ANT was also expresses containing the PLB sequence, H6-wtPLB-ANT (Seq. ID. No. 17), in addition to an H6-PLB(S16E mutant)-ANT protein and an H6-PLB (V49A mutant)-ANT protein (Seq. ID. Nos. 18 and 19 respectively). H6-beta-galactosidase-

25   ANT, H6-TAT, and H6-beta-galactosidase-TAT were also expressed at lower levels (seq. not listed). A non-functional ANT-penetratin with two mutations at residues 68 and 67, where Trp was mutated to Phe were made as negative control for the other three penetratin-PLB proteins.

To measure the effect these recombinant penetratin-based proteins

30   have on cardiac contraction, one mouse was injected intraperitoneally

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with 2 mg of the H6-ANT peptide. A second mouse was injected
intraperitoneally with 2 mg of the H6-ANT mutant protein. After a 3 hour
incubation period, the mice were sacrificed and the hearts removed for
analysis. The blood in the heart was removed by forcing fluid backwards

5   through the aortic arch. Each heart was dissected into atrial tissue, left
ventricular tissue, and right ventricular tissue. All the tissue was washed
extensively in a physiologically balanced PBS solution and flash frozen in
liquid nitrogen. The tissue were then lysed in 8 M Urea, 2% triton-X100,
for 10 minutes and equal amounts of the supernatants were

10   electrophoresed on 15% PAGE. The bands were transferred to a PVDF
membrane. The membranes were labeled with anti-His antibody in order
to identify if the lysate contained the epitope tagged protein.

The invention disclosed herein provides several methods for the
treatment of heart failure through the inhibition or alteration of the

15   interaction between phospholamban and sarcoplasmic reticulum Ca^{2+} -
ATPase within the cardiomyocytes. Although the invention has been
described with reference to the examples provided above, it should be
understood that various modifications can be made without departing
from the spirit of the invention. Accordingly, the invention is limited only

20   by the following claims.

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What is claimed is:

1.   A method for treatment of heart failure comprising inducing phospholamban deficiency.

2.   The method for treatment of heart failure of claim 1, wherein an exogenous phospholamban protein induces phospholamban deficiency.

3.   The method for treatment of heart failure of claim 2, wherein the exogenous phospholamban protein is selected from the group consisting of mutations of PLB, sense PLB, antisense PLB, truncated PLB, native PLB, and antibody against PLB.

4.   The method for treatment of heart failure of claim 3, wherein the mutations of PLB comprise point mutations of PLB.

5.   The method for treatment of heart failure of claim 3, wherein the antibody against PLB comprises contractilin.

6.   A peptide based therapeutic agent for inhibiting phospholamban activity consisting of a first peptide and a second peptide as a complex, wherein the first peptide comprises a transport peptide and the second peptide comprises a cargo peptide.

7.   The peptide based therapeutic agent of claim 6, wherein the transport peptide is selected from the group consisting of penetratin, adenovirus , bacterial and lipid vesicle based transport peptide.

8.   The peptide based therapeutic agent of claim 6, wherein the cargo peptide is selected from the group consisting of mutations of PLB, sense PLB, antisense PLB, truncated PLB, and native PLB protein.

9.   The peptide based therapeutic of claim 6, wherein the first peptide transports the second peptide across a cell membrane.

10.   The peptide based therapeutic of claim 6, wherein the first peptide and the second peptide are linked by a covalent linkage.

11.   The peptide based therapeutic of claim 10, wherein the covalent linkage consists of a branched polylysine backbone, a single peptide bond, or a disulfide bond.

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12. A method for treatment of heart failure comprising enhancement of cardiac contractility by inhibition of PLB-SERCA2a interaction.

13. The method of claim 12, wherein the cardiac contractility is

5 enhanced by inhibiting effect of PLB on sarcoplasmic reticulum Ca^{2+} ATPase.

14. The method of claim 12, wherein an exogenous phospholamban protein is used to inhibit phospholamban deficiency.

15. The method of claim 14, wherein the exogenous

10 phospholamban protein is selected from the group consisting of mutations of PLB, sense PLB, antisense PLB, truncated PLB, native PLB, and antibody against PLB.

16. The method of claim 15, wherein the mutations of PLB comprise point mutations of PLB.

15 17. The method for treatment of heart failure of claim 15, wherein the antibody against PLB comprises contractilin.
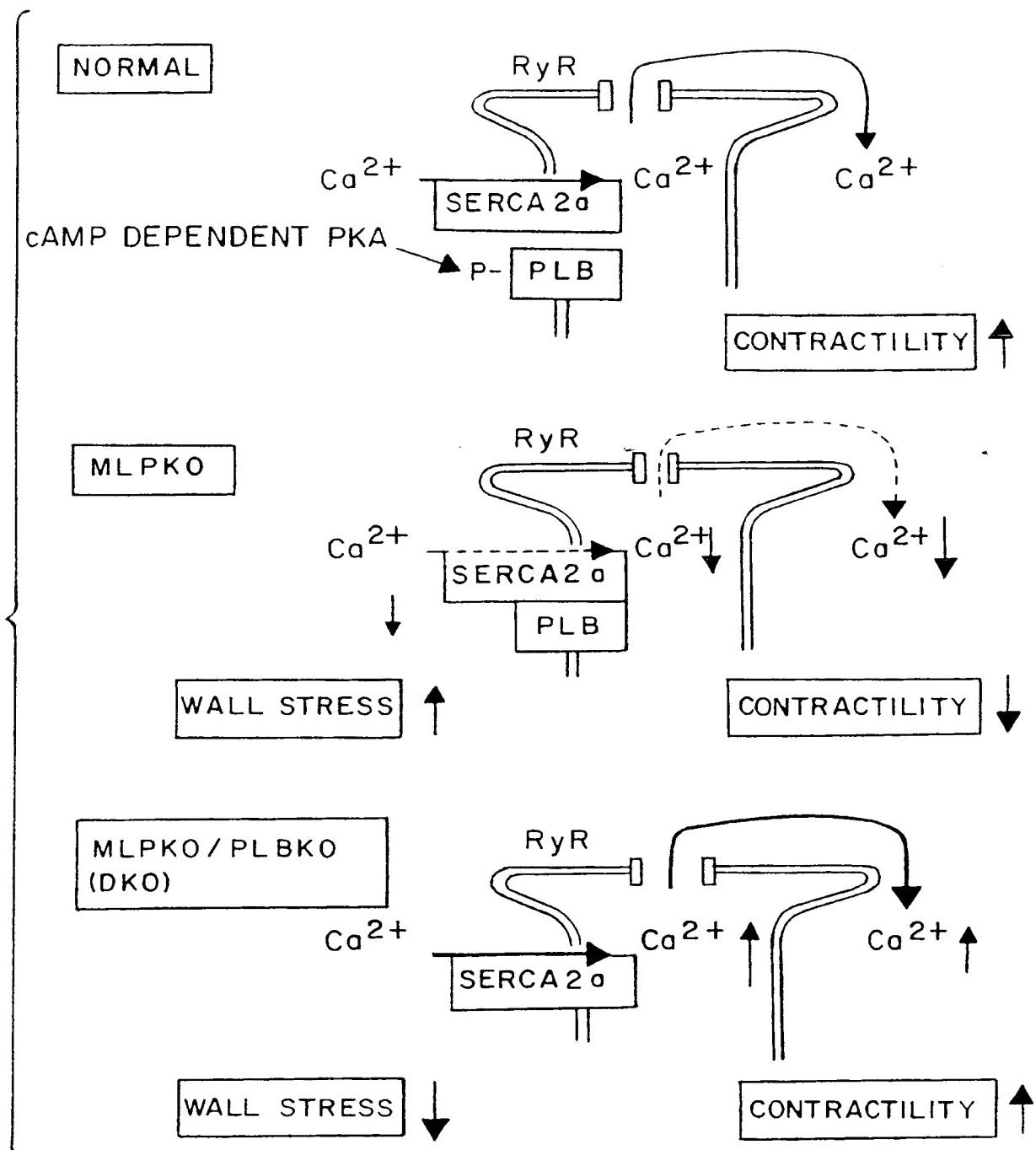
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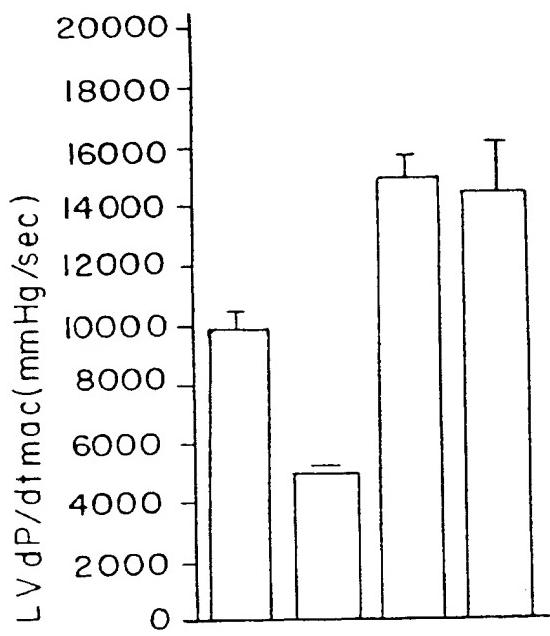
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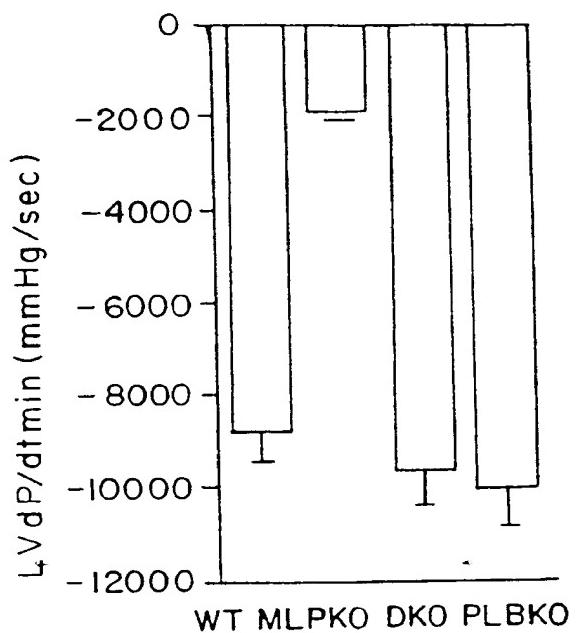
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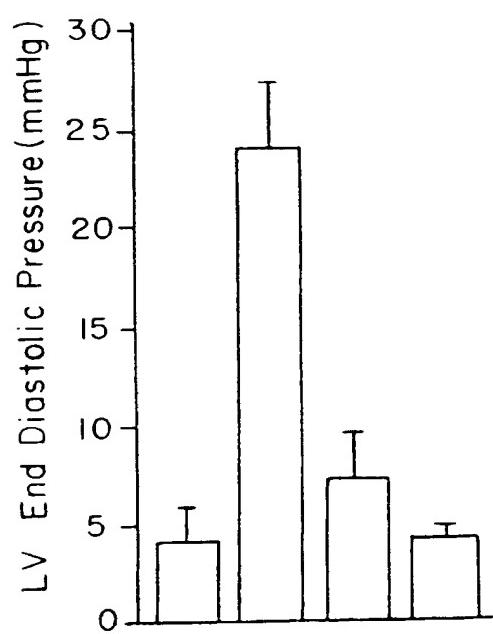
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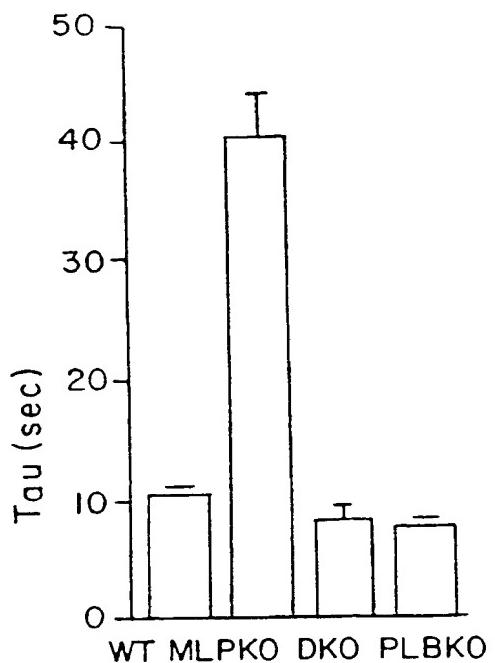
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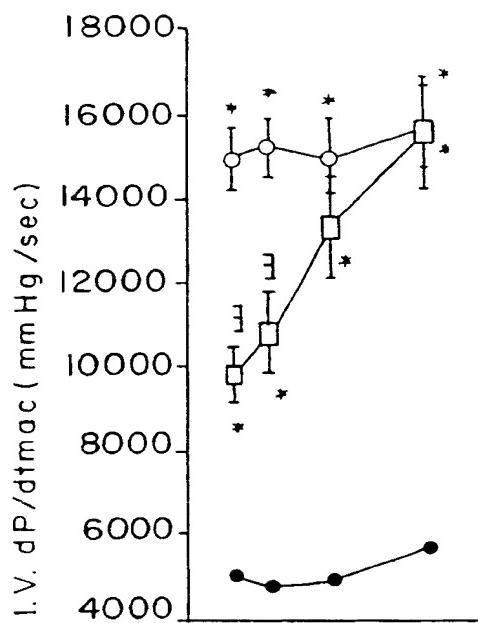
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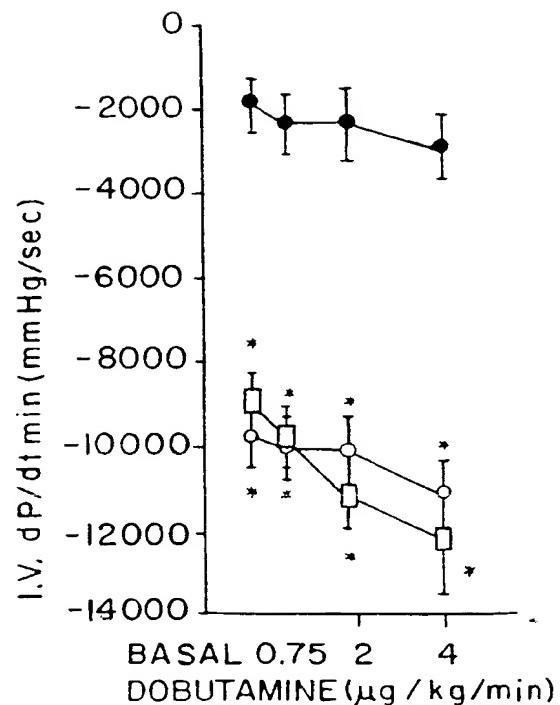
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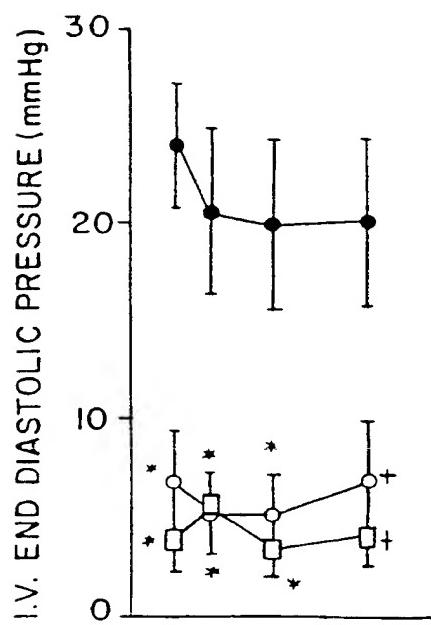
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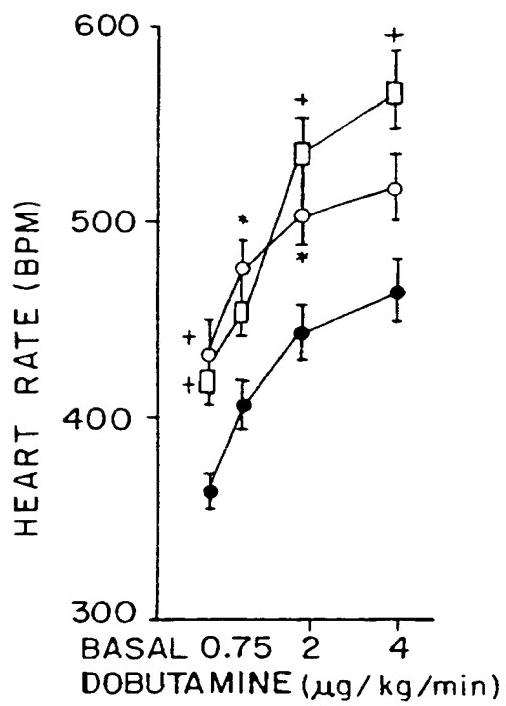
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4/9
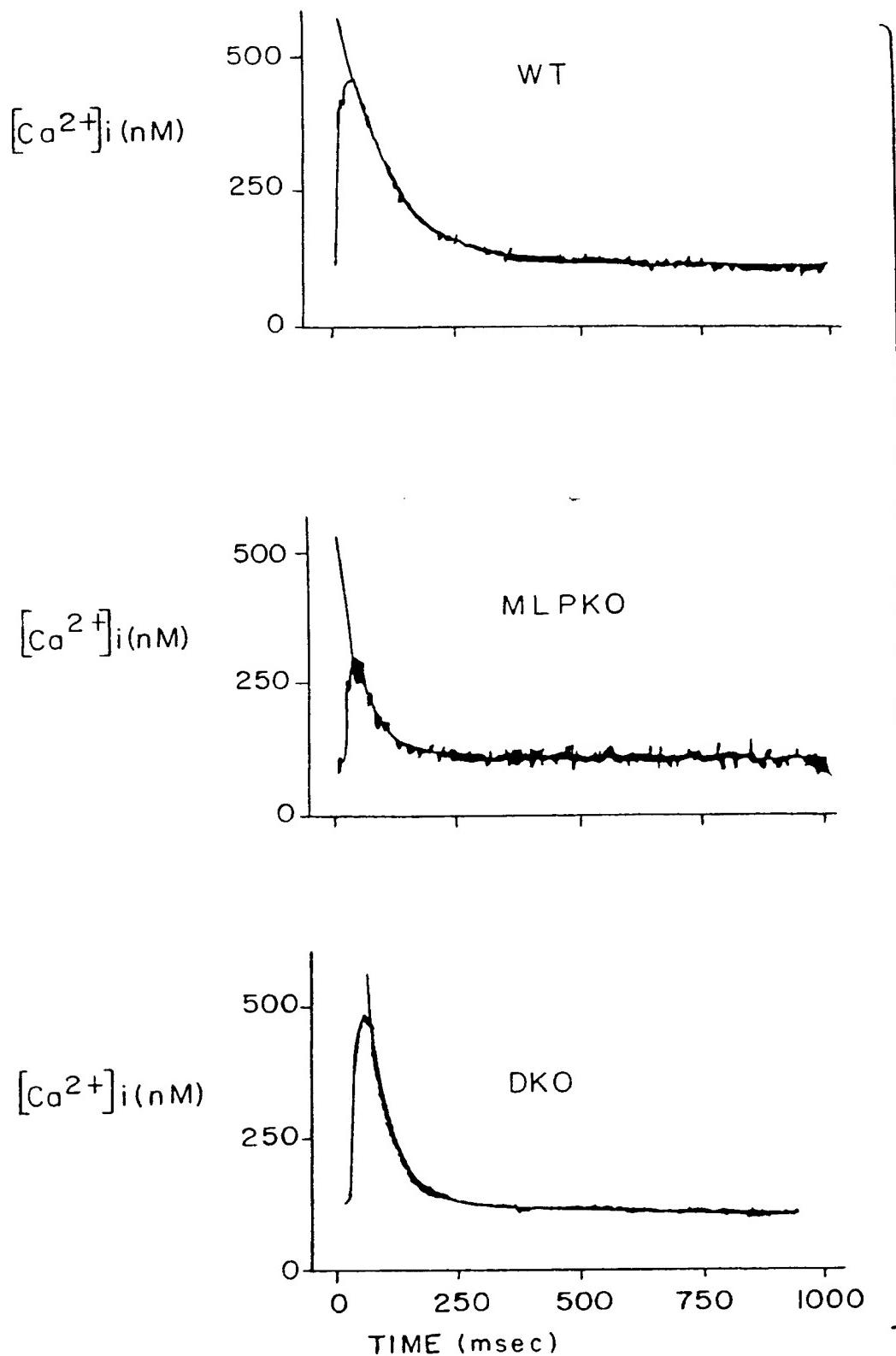
FIG. 3a

5/9
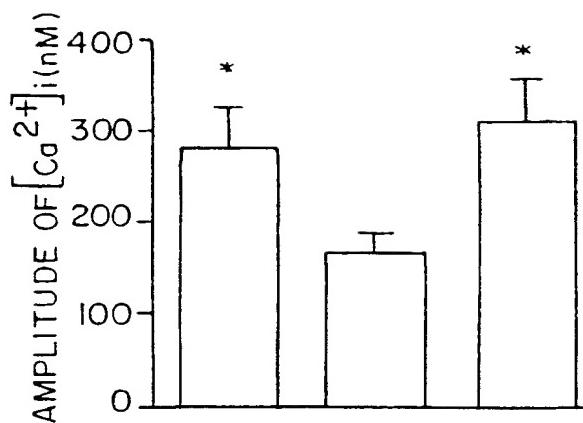
FIG. 3 b

FIG. 3c

FIG. 3d

FIG. 4b

FIG. 3e

FIG. 4a

FIG. 5a

	CONTROL n=7		WILDTYPE PLB n=3		V49A MUTANT PLB n=4	
SHORTENING %	7.20	0.60	3.50	0.24 *	11.45	0.88 **
-dL/dt (mm)	88.4	10.3	48.4	5.9 *	150.3	9.6 **†
+dL/dt (mm)	56.6	6.5	28.1	6.4†	116.5	13.5 **†
CELL LENGTH(μm)	104.5	3.1	109.2	3.9	108.7	3.3

FIG. 5b

MARKER(kD)

SR(-) sPLB asPLB E2A K3E/RI4E SI6N RI4E

43 —

29 —

6.2 —

← α-ACTIN
← (PLB/mPLB)6
← PLB4

← PLB I

FIG. 6a

sPLB K3E/RI4E sPLB+K3E/RI4E

FIG. 6b

← (PLB/mPLB)s
← PLBs

LacZ sPLB K3E/RI4E sPLB+K3E/RI4E

MARKER (kD)

43 —

29 —

18 —

12 —

← mPLBs
← PLBs

← PLB I

FIG. 6c

FIG. 7

FIG.8

SEQUENCE LISTING

<110>   Kenneth Chien, Wolfgang Dillman, Susumu Minamisawa,
           Huaping He, Masahiko Hoshijima, Markus Meyer, Christopher
           Scott, Yibin Wang, Gregg Silverman

<120>   A METHOD FOR INHIBITION OF PHOSPHOLAMBAN
           ACTIVITY FOR THE TREATMENT OF CARDIAC DISEASE
           AND HEART FAILURE
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<140>   unknown
<141>   November 2, 1999
<150>   US 60/106,718
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      20   25   30   35

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        20                  25                  30                  35


Tyr Ala Gly Ser Tyr Tyr Tyr Gly Trp Phe Gln Gln Lys Ser Pro Gly Ser Ala
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                    40   45   50

Pro Val Thr Val lle Tyr Ser Asn Asp Gln Arg Pro Ser Asn lle Pro Ser Arg
55   60   65   70

Phe Ser Gly Ser Thr Ser Gly Ser Thr Ser Thr Leu Thr lleThr Gly Val Arg
        75   80   85   90

Ala Glu Asp Glu Ala Val Tyr Phe Cys Gly Ser Asn Ser Gly Thr Gly Tyr Val
            95   100   105

Gly lle Phe Gly Ala Gly Thr Thr Leu Thr Val Leu Gly Gln Ser Ser Arg Ser
    110   115   120   125

Ser Thr Val Thr Leu Asp Glu Ser Gly Gly Gly Leu Gln Thr Pro Gly Gly Ala
        130   135   140

Leu Ser Leu Val Cys Arg Ala Ser Gly Phe Thr Phe Ser Arg Phe His Met
145   150   155   160

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            165   170   175

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180   185   190   195

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            200   205   210

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215   220   225   230

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Ala Ser
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            20   25   30

Lys Asp Pro Ser Ser Arg Ser Ala Ala Gly Thr Met Glu Phe Arg
            35   40   45

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            50   55   60

Ala
61

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 1   5   10   15

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            20   25   30

Leu Phe Ile Asn Phe Cys Leu Ile Leu Ile Cys Leu Leu Leu Ile
          35   40   45

Cys Ile Ile Val Met Leu Leu His His His His His His Lys Gly
        50   55   60

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          65   70   75

Trp Lys Lys Ala
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        20   25   30

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          35   40   45

Cys Ile Ile Val Met Leu Leu His His His His His His Lys Gly
        50   55   60

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                20   25   30

Leu Phe Ile Asn Phe Cys Leu Ile Leu Ile Cys Leu Leu Leu Ile
                35   40   45

Cys Ile Ile Ala Met Leu Leu His His His His His His Lys Gly
                50   55   60

Glu Phe Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys
                65   70   75

Trp Lys Lys Ala
                79